A LIVING HISTORY OF IMMUNOLOGY

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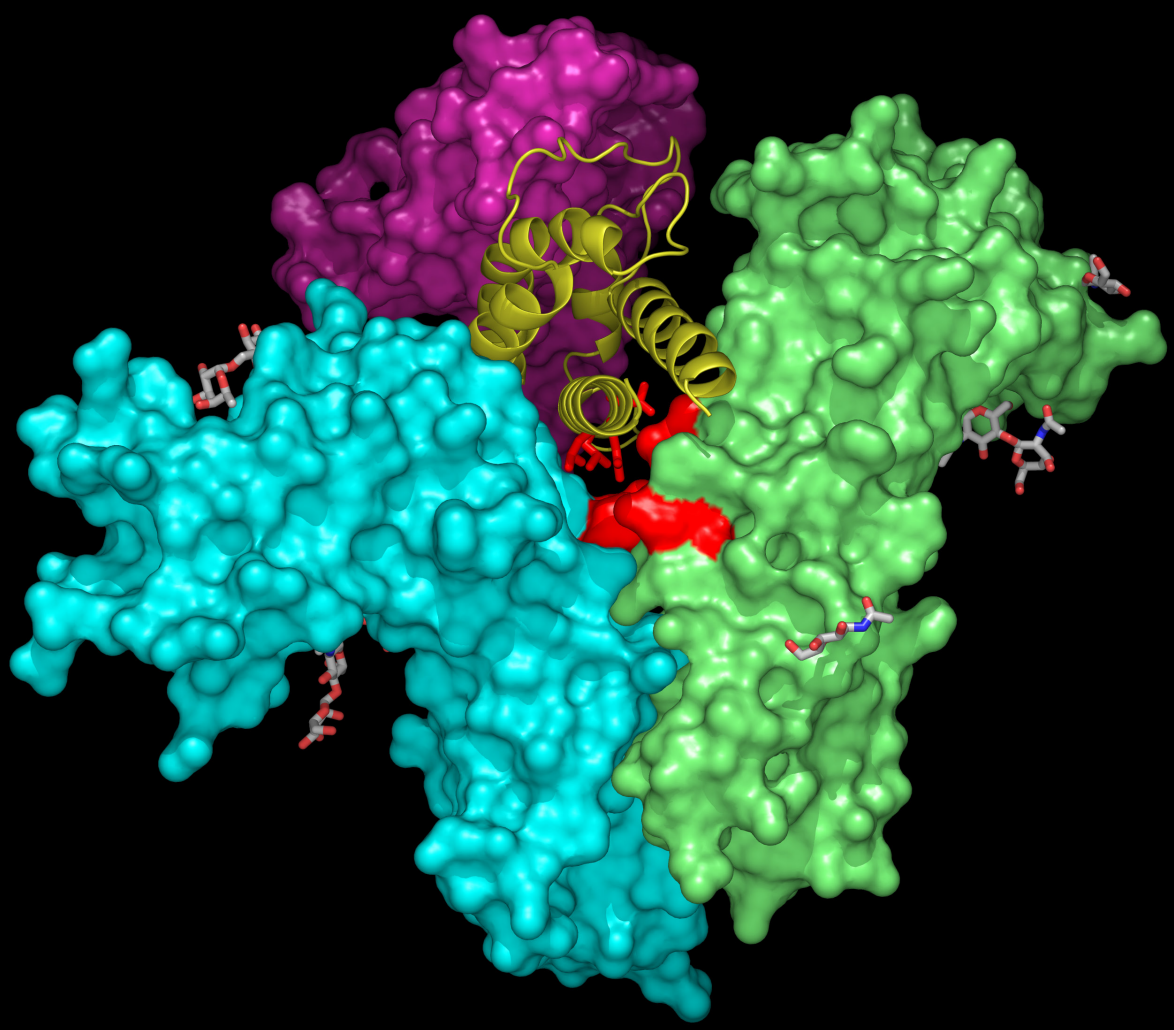

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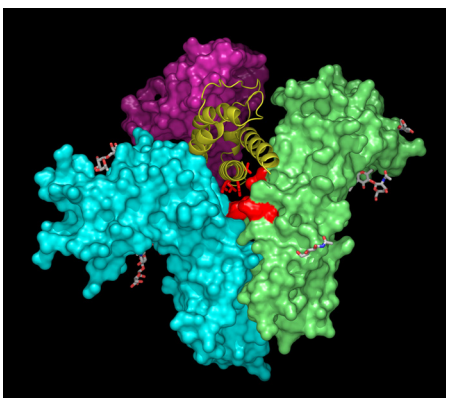
A LIVING HISTORY OF IMMUNOLOGY

Topic Editor:
Kendall Arthur Smith, Cornell University, USA



Crystal structure of the heterotrimeric interleukin-2 receptor (IL-2Rαβγ) in complex with IL-2. IL-2Rβ (cyan) and IL-2Rγ (green) form a three-way junction with IL-2 (yellow) at the heart of the quaternary IL-2 signaling complex. The network of residues that mediate these contacts (colored red) provides a compelling structural basis for cooperativity in the IL-2/IL-2 receptor complex assembly. IL-2Rα (magenta) makes no contacts with IL-2Rβ or γc, supporting its principal role to deliver IL-2 to the signaling complex and act as regulator of signal transduction. Carbohydrates are displayed as ball-and-stick models. Image by Erik W. Debler, Rockefeller University.

In the highly competitive world of biomedical science, often the rush to publish and to be recognized as "first" with a new discovery, concept or method, is lost in the hurly-burly of the moment, as "the maddening crowd" moves on to the next "new thing". One of the great things about immunology today is that it has only become mature as a science within the last half-century, and especially within the past 35 years as a consequence of the revolution of molecular immunology, which has taken place only since 1980. Consequently, most of those who have contributed to our new understanding of how the immune system functions are still alive and well, and still contributing. Thus, "A Living History of Immunology" collates many stories from the investigators who actually performed the experiments that have established the frontiers of immunology. Accordingly, this volume combats "revisionist science", by those who want to alter history by telling the stories a different way than actually happened. In this regard, one of the good things about science vs. other disciplines is that we have the written record of what was done, when it was done and by whom. Even so, we do not have the complete story or narrative of how and why experiments were done, and what made the differences that led to success. This volume captures and chronicles some of these stories from the past fifty years in immunology.

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EDITORIAL
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Editorial: A living history of immunology

*Kendall A. Smith**

Division of Immunology, Department of Medicine, Weill Medical College, Cornell University, New York, NY, USA

Keywords: immunological history, adaptive immunity history, interleukins, cytokines, lymphokines, T cell antigen receptors

This Research Topic was conceived to provide a venue for investigators to document their critical contributions to our understanding of the cellular and molecular mechanisms that provide our remarkable immune system the capacity to protect us from environmental insults while simultaneously remaining unreactive to our internal molecular milieu. Although the scientific literature gives one a history of what happened, when it happened, and who were responsible, it fails to capture precisely how things came together, and why some investigators were successful, while others working contemporaneously failed. Thus, seminal contributors were asked to recount the various aspects of their experiments and people who were instrumental in making the progress that moved our understanding forward.

Looking back over the brief history of immunology, a discipline that arose after the pioneering approaches of Edward Jenner introduced the smallpox vaccine in 1798 (1) and Louis Pasteur catapulted immunology into universal awareness 100 years later (2–4), the science has only become mature within the past 50 years. For almost 100 years after Pasteur, experimentalists focused on observations of the reactions of whole experimental animals or humans to the administration of putative antigenic substances. For the first time, around 1960, it was appreciated that lymphocytes are the cells that mediate the immune reaction (5–7), and experimentation moved for the first time from *in vivo* to *in vitro*, which allowed one to manipulate and investigate an immune reaction of cell populations "outside of the black box." During the 1960s, various techniques were improved so that it was possible to discern that several different types of cells cooperated to ultimately generate a measurable immune response, usually monitored by the appearance of antibody-forming cells (AFCs) (8, 9).

As detailed in this Research Topic, by the 1970s, experiments culminated in the demonstration that two distinct types of lymphocytes existed, termed thymic-derived cells (T cells) and bone marrow-derived cells (B cells), which generate AFCs. Furthermore, a third type of cell derived from myeloid cells, termed an antigen-presenting cell (APC), also played a role. Evidence was also presented that there are at least two distinct subsets of T cells. Moreover, investigators detected activities in culture supernatants of activated lymphocyte populations that seemed to enhance or suppress the generation of AFCs as well as the proliferation of various lymphocytes. However, the molecular basis of these activities remained enigmatic and essentially unapproachable, given the experimental biochemical methods then available.

Four new and novel experimental methods were introduced in the 1970s that revolutionized all of biological sciences, which enabled a further reduction from cells to molecules, and led to the discipline that now can be recognized as molecular immunology. In 1972, the flow cytometer introduced a new method to identify and isolate individual cells present in cell populations (10). Also, genetic engineering approaches enabled investigators to identify and isolate complimentary DNA molecules encoding gene products, which then allowed the ready determination of their primary structures, and provided a means to generate essentially unlimited quantities of critical proteins (11). Third, the advent in 1975 of the capacity to isolate and clone individual AFCs, which

Edited and reviewed by:
Paulo Vieira,
Institut Pasteur de Paris, France

***Correspondence:**
Kendall A. Smith
kasmith@med.cornell.edu

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could produce unlimited quantities of monoclonal antibody molecules (12), could then be used to identify and isolate both individual cells using the flow cytometer, but also new reagents that could be used to isolate and purify individual protein molecules from complex mixtures.

The fourth critical technical advance, which like monoclonal antibodies was also special to immunology, was the creation in 1979 of the methods to select, clone, and grow the functional progeny of individual T cells (13). This advance, for the first time, allowed one to circumvent the tremendous heterogeneity of individual cells within T cell populations so that the molecules responsible for antigen recognition, histocompatibility restriction, and the molecular mechanisms underlying T cell function, including T cell help of antibody production and T cell mediated cytolysis, could be uncovered.

The contributions comprising this compilation of the stories about how the transition from experiments on whole living organisms to cell populations to individual cells and finally to homogeneous molecules represent a unique aspect of scientific communication, in that they tell the behind the scenes dramas that are usually left out of the scientific literature. This Research Topic tells how science gets done, and what the people are like who actually do it. I hope that you enjoy the stories.

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Revisiting thymus function

Jacques F. A. P. Miller*

Department of Immunology, Walter and Eliza Hall Institute of Medical Research, Parkville, VIC, Australia
**Correspondence: miller@wehi.edu.au*

Edited by:
Nick Gascoigne, National University of Singapore, Singapore

Reviewed by:
Charles Surh, The Scripps Research Institute, USA
Nick Gascoigne, National University of Singapore, Singapore

Keywords: thymus, thymic-dependent lymphocytes, adaptive immunity, cellular immunity, neonatal thymic function

For centuries, the thymus has been an organ in search of a function. The fact that it is a large mass of tissue in infancy was not appreciated at the beginning of the twentieth Century, as autopsies performed in infants succumbing to fatal illnesses such as diphtheria, revealed a small thymus. This resulted from stress during the illness, but the small size of the thymus was thought to be the norm. When infant death occurred during anesthesia for stress-unrelated conditions, fatality was blamed not on the anesthetic but on the large thymus. Some doctors even prescribed radiation therapy to shrink the thymus (1), not realizing that some of their patients would later develop adenocarcinoma of the thyroid.

Prior to 1961, the thymus was considered not to have any role in immunity. The major reasons for this can be summed up as follows. Unlike lymphocytes obtained by thoracic duct cannulation or from spleen and lymph nodes, thymus lymphocytes were generally poor in their ability to initiate immune reactions after adoptive transfer to appropriate recipients. Thoracic duct lymphocytes could home from blood into lymphoid tissues, "the only exception" being "the thymus in which very few small lymphocytes" appeared "to lodge" (2). The production of antibody-forming plasma cells and the formation of germinal centers, so prominent in spleen and lymph nodes, were not seen in thymus tissue of normal or immunized animals. Defects in immune responsiveness had never been documented in mice whose thymuses had been removed during adult life, a fact that had led some groups to conclude that "the thymus gland does not participate in the control of the immune response" (3). At a Symposium on Cellular Aspects of Immunity (4), in which took part world-renowned immunologists including Burnet, Good, Lederberg, Medawar, and Mitchison, and published in 1960, not a single reference was made to the thymus or to its cells throughout the meeting. Immunologists believed that, as a predominantly epithelial organ, the thymus had become vestigial during evolution and was just a graveyard for dying lymphocytes. Medawar even stated, "We shall come the regard the presence of lymphocytes in the thymus as an evolutionary accident of no very great significance" (5).

In the late 1950s, I was working on mice with lymphocytic leukemia that was induced in low-leukemic strain mice [as demonstrated by Ludwik Gross (6)] by injecting filtered extracts of leukemic tissues obtained from high leukemic strain mice. A leukemogenic virus was believed to be the causative agent and it had to be given to newborn mice to obtain a high incidence of leukemia. The disease began in the thymus and thymectomy at 1 month of age prevented its onset (7). Grafting a neonatal thymus 6 months after thymectomy restored the potential for leukemia development (8), and the virus could be recovered from the non-leukemic tissues of thymectomized mice (9). But why did it have to be given at birth? One possibility was that it could multiply only in neonatal thymus and would then spread to other sites. To test this, mice were thymectomized before the virus was given and therefore at birth.

The survivors grew well at first but, after weaning, many wasted and died prematurely whether inoculated with virus or not. Adult thymectomy, on the other hand, had never shown any untoward effects such as weight loss or obvious pathology. This led me to conclude "that the thymus at birth may be essential to life" (10). Histological examination of the tissues of neonatally thymectomized mice revealed a marked deficiency of lymphocytes in the circulation and the lymphoid tissues and many wasted mice had liver lesions suggesting infection by some hepatitis virus (11, 12). At that time Gowans had shown that small lymphocytes were not short lived cells, as had been thought before, but immunologically competent cells with a long lifespan, recirculating from blood through lymphoid tissues into lymph and able to initiate immunological reactions when appropriately stimulated by antigen (13). Clearly, my neonatally thymectomized mice must have been immunodeficient, which accounted for their susceptibility to virus infections. I therefore tested their immune competence by grafting skin from allogeneic mice and from rats. The results were incredibly spectacular and published first in The Lancet in 1961 (11) and in greater detail in the Proc Roy Soc. (12). The mice failed to reject skin both from totally unrelated strains ("H-2-incompatible") and from rats, and failed to do so even when grafted before the onset of wasting. Since both Gowans and Medawar had firmly established that rejection of foreign skin grafts was mediated by lymphocytes, and since my mice were deficient in lymphocytes following neonatal thymectomy, it was logical for me to conclude that the thymus was the source of immunologically competent lymphocytes, at least during the neonatal period. Contrary to the prevailing opinion, I postulated "during embryogenesis the thymus would produce the originators of immunologically competent

cells many of which would have migrated to other sites at about the time of birth. This would suggest that lymphocytes leaving the thymus are specially selected cells" (11). I had therefore proposed the bold postulate that the thymus was the site responsible for the development of immunologically competent small lymphocytes.

The few neonatally thymectomized mice that did eventually reject allogeneic skin grafts were later grafted again with skin from the same donors but showed no evidence of a second set response (12). By contrast, neonatally thymectomized mice bearing well-established allogeneic skin rejected that skin rapidly when given intravenous lymphocytes from normal donors that had been immunized to skin of the same strain (12).

I tested the ability of my neonatally thymectomized mice to produce antibody to *Salmonella typhi* H antigen and found this to be impaired (12).

Grafting thymus tissue to neonatally thymectomized mice prevented immunological deficiency. Although implantation of syngeneic thymus tissue allowed these mice to develop a normal immune system, grafting a thymus derived from a foreign strain induced specific immune tolerance to the histocompatibility antigens of the donor. Thus, lymphocytes developing in the thymus in the presence of foreign cells must have been deleted [i.e., "selectively thymectomized" as I suggested (12)]. Hence, by implication, the thymus should be the site where self tolerance is imposed and where discrimination between self and non-self takes place.

Showing that cells from the thymus migrated into the lymphoid tissues was difficult at that time, since no markers had been found to identify cells from different locations. So I made use of the T6 mouse strain the cells of which could easily be identified at metaphase by the presence of 2 min chromosomes. Neonatally thymectomized F1 hybrid mice in which one parent was T6, were grafted with thymus from the other parental strain and immunized with skin from various donors. An analysis of the chromosome constitution of the cells in metaphase in the spleen showed that 15–20% had originated from the thymus graft (12).

My conclusions concerning the immunological function of the thymus were regarded with skepticism by the immunological community. For example, Medawar was not convinced as evident from a letter he sent to me in which he wrote: "I take it that the thymic tissue seen in fishes is wholly or predominantly epithelial, as its phylogenetic origin suggests. It is a matter of some interest that many organs, which seem to become redundant in the course of evolution undergo a sort of lymphocytic transformation" (14). Trivial criticisms abounded: what I had observed must surely have occurred only in the strain of mice that I had been using; my mice must have been in such poor health that any surgical trauma would prejudice their ability to reject skin grafts; whatever the thymus might have been doing in my mice, it could not possibly do in humans! At a Ciba Foundation Symposium on Tumor Viruses of Murine Origin held in Perugia in June 1961, the first international meeting where I presented my results, R.J.C. Harris, claimed the following: "Dr. Delphine Parrott in our laboratory has been thymectomizing day-old mice and there is at present no evidence that these animals are immunologically weaker than normal animals. They do not retain skin grafts; they are living and breeding quite normally. They do not die of laboratory infections" (15).

These criticisms did not last very long as I and several other researchers repeated, confirmed, and extended my results. It was evident, for example, that the adult thymus would still play a role in immunogenesis and this was shown when the rest of the lymphoid system was destroyed by total body irradiation and the mouse protected by an injection of bone marrow (16, 17). The adult thymectomized irradiated and marrow protected mice were crucial to our subsequent demonstration of the existence of two major lymphocyte subsets, T and B cells (18). An avalanche of work followed these early investigations.

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OPINION ARTICLE
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On discovering thymus–marrow synergism

Henry N. Claman [1,2] *

[1] Department of Medicine, University of Colorado School of Medicine, Denver, CO, USA
[2] Department of Immunology, University of Colorado School of Medicine, Denver, CO, USA
*Correspondence: henry.claman@ucdenver.edu

Edited by:
Kendall A. Smith, Weill Medical College of Cornell University, USA

Reviewed by:
Janet M. Stavnezer, University of Massachusetts Medical School, USA
Michael R. Gold, The University of British Columbia, Canada
John William Schrader, The University of British Columbia, Canada

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In the 1960s, the thymus was an organ of mystery. Although it was full of lymphocytes, they made no antibodies. Furthermore, thymectomy (in mature animals) failed to produce immunological inadequacy. This situation changed when J.F.A.P. Miller did *neonatal thymectomy*, which was indeed followed by a syndrome including crippling of the immune response (1–3).

The possible role of the thymus was the focus of a several-day symposium organized by Robert A. Good in November, 1962 in Minneapolis. Its proceedings, *The Thymus in Immunobiology*, summarized the clinical and experimental data (4).

This mystery organ intrigued me. Systemically, immunized animals did not make antibody in the thymus as they did in lymph nodes or spleen. Maybe, we thought, there was some kind of blood–thymus barrier, which prevented systemic antigen from interacting with thymocytes in the thymic parenchyma. We used adoptive transfer of syngeneic cells into irradiated recipients. In this model, spleen cell suspensions responded to sheep erythrocyte (sheep red blood cells, SRBC) antigens by making hemolytic antibody in the recipient spleens and serum. Would thymus cell suspensions similarly prepared (so as to break any blood–thymus barrier) do the same?

I was a young investigator working in the late David W. Talmage's lab at the University of Colorado Medical School in Denver. It was a stimulating environment. Edward A. Chaperon and R. Faser Triplett were post-doctoral fellows. On day 0, the mice were irradiated and then injected i.v. with spleen cells or thymus cells. On day 1, we injected the SRBC antigen IV. On day 5, we sacrificed the mice and looked for anti-SRBC-producing cells in the recipient spleens. The results were clear-cut. Recipients of donor spleen cells made many antibody-producing cells while recipients of donor thymus cells did not. Perhaps, we thought, 4-days of exposure to antigen after transfer might have been sufficient to get the mature spleen cells to make antibody but insufficient for the (putatively) immature thymocytes to do the same. We needed to lengthen the protocol.

The next experiments were identical on days 0 and 1, but on day-4 recipients got a booster injection of SRBC antigen, and we planned to sacrifice on day 8. This worked well in the group that received spleen cells but the recipients of thymus cells (the test group) were all dead by day 8. We figured that this represented radiation death in the thymus recipients, whereas the spleen recipients survived because of the hematopoietic stem cells in the inoculum.

We knew of the radio-protective effects of bone marrow cells, so it made sense to add an aliquot of such cells to the thymus inoculum. Indeed, the recipients of thymus-plus-bone marrow survived until day 8, but to our surprise, these recipients produced almost as much antibody as did spleen recipients. (Later we added a new group as another control, i.e., bone marrow cells only. They caused no significant antibody production.)

We called this phenomenon "thymus–marrow synergism," and it was the first demonstration that two (presumably lymphoid) cell populations were needed for significant antibody production. We speculated that one sort of cell (the "effector") made the antibody while another variety of cell from the other inoculum performed in an "auxiliary" mode. On the basis of indirect evidence, we postulated that the bone marrow provided the effector cells and the thymus cells were "auxiliary." Support for this view had to await the definitive experiments by others.

Our experiments were published in 1966 in Proc. Soc. Exptl. Biol. Med. (5). The paper was widely acknowledged to have demonstrated cell–cell interaction in the antibody response. Additional findings by the three of us were considered important enough many years later when they were chosen as the first article to be mentioned in the *Journal of Immunology's* new historical series, Pillars of Immunology.

This discovery was unexpected – almost representing serendipity. Not everyone was convinced. However, others used the paradigm to provide further elucidation of the mechanism of thymus–marrow synergism. Mitchell and Miller made great progress by identifying the antibody-forming cell as originating in the bone marrow (6). Additionally, Avrion Mitchison added the brilliant insight that *the carrier effect* was an example of T–B collaboration where the anti-hapten antibody was made by bone marrow-derived cells while the thymus-derived cells provided "help" (7–9).

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In vitro studies of the antibody response: antibodies of different specificity are made in different populations of cells

Richard W. Dutton*

Pathology Department, University of Massachusetts Medical School, Worcester, MA, USA
**Correspondence: Richard.Dutton@umassmed.edu*

Edited by:
Kendall A. Smith, Weill Medical College of Cornell University, USA

Reviewed by:
Ellis L. Reinherz, Dana-Farber Cancer Institute, USA
Nick Gascoigne, National University of Singapore, Singapore

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Our paper (1) "Cell populations and cell proliferation in the *in vitro* response of normal mouse spleen to heterologous erythrocytes. Analysis by the hot pulse technique," investigated the kinetics of antigen driven proliferation of antibody forming cells in culture and showed that different populations of cells were involved in the response to two different antigens, providing evidence for the clonal selection theory proposed by both Sir McFarlane Burnet and by David Talmage.

I was trained as a biochemist in England but switched to immunology when I was given the opportunity to come to the United States to join John Vaughan's lab at the Medical College of Virginia in Richmond, Virginia. I arrived in January 1957 and was soon immersed in the great debates then raging in immunology.

At that time, the revolution in biology was well underway. The double helix model for DNA was propounded in 1953 but it took some time for the significance of the double helix to sink in. The "central dogma that DNA codes for RNA that codes for protein" came later and the nature of the genetic code and the mechanisms of protein synthesis (messenger RNA, transfer RNA, and ribosomes) were not completely worked out until 1964–66, more than 10 years later.

Immunology, however, was a separate arena. In 1957, most experiments in immunology were carried out in whole animals. Antigen in, wait 10 days, antibody out, and everything in between took place in a black box. The structure of the antibody molecule, immunoglobulin genes, cytokines, signaling pathways, and almost all of what we now study was all unknown.

It was thought that the architecture of the lymphoid organs was essential to their proper function (and indeed, we are just now coming back to that same understanding) and it worried people that half the spleen cells put out into culture were dead in 24 h. As a biochemist, accustomed to the study of metabolism in cell free fractions, I was not deterred and pushed ahead regardless. Our first goal was to get immune responses from single cell suspensions *in vitro* where we could study them and manipulate them under various conditions.

At that time, there was no *in vitro* system that did anything other than just demonstrate that antibody was being made and most employed tissue slices or fragments rather than single cell suspensions (2). The trick that led to our success was to start the response *in vivo* and then switch to culture and continue the response *in vitro*. The assay for antibody was the measurement of incorporation of radiolabeled amino acid into antibody that could be recovered by coprecipitation with antigen antibody complexes (2). Using this, we could quantitate the rate of antibody synthesis and measure the kinetics of the response and determine how it was affected by culture conditions. Our first application was to investigate the metabolic activity of antibody formation and to show that the incorporation radiolabeled phosphate into acid soluble, fat soluble, RNA, and DNA phosphate was increased in antibody forming cultures. From today's perspective, this was not the most obvious thing to do but many investigators were conducting similar analyses in the study of protein synthesis. People looked on antibody synthesis in lymphoid organs in the same way that they looked on albumin synthesis in the liver. The protein was assumed to be made in all the cells of the organ, not as the product of small but rapidly expanding subset of the cells, and it seemed that the global analysis of the accompanying biochemical events might be revealing. This proved not to be the case but our focus was soon switched to more profitable studies.

We went that April, to the Symposium on "Antibodies: Their production and mechanism of action" sponsored by the Biology Division of the Oak Ridge National Laboratory, Oak Ridge, Tennessee. Presentations from the meeting were published in the Journal of Cellular and Comparative Physiology Volume 50, Supplement 1, December 1957 and contained papers by Frank Dixon, Jon Singer, Elvin Kabat, David Talmage, and N.A. (Av) Mitchison, to mention just a few that may still be known to the "elders" in our field. Also, presented were two papers by "young Turks," one by Novelli and DeMoss (3) and the other by Schweet and Owen (4), both of which sought to apply the new understanding of the molecular biology of the control of protein synthesis to the synthesis of antibody. They received only a mixed reception from the old school immunologists, brought up in a discipline still isolated from the main body of biology.

After the meeting, we made our way by car to the annual AAI/FASEB meeting that year in Chicago but bumped into Talmage and his family in the caverns at Mammoth Caves in Kentucky, which led to an invitation to visit him in his house while at the meeting in Chicago. There, in the crowded kitchen, Talmage, Burnet, Vaughan, and others engaged in a vigorous argument of the pros and cons of the, not yet quite crystallized, clonal selection theory (5, 6), that stated the individual antibody forming cells were committed to the synthesis of just one unique antibody. The theory allowed one to explain many aspects of the induction of immunological tolerance and generated great excitement at the time.

In the years that followed, the clonal selection theory became generally accepted, based more on its intellectual appeal rather than on experimental evidence, which was actually somewhat conflicting.

Our contribution (1) in support of the theory was to show that cells making one antibody could be destroyed using a "hot pulse" technique without affecting a second population in the same culture that were making another antibody.

The lead-up to this began years earlier when we showed, in 1958, that an *in vitro* antibody response could be drastically reduced by 8-azaguaninne, an inhibitor of RNA and DNA synthesis (7). Burnet had suggested that the induction of antibody synthesis might be analogous to the induction of inducible enzymes (8) and Creaser had shown that inducible enzyme synthesis in bacteria could be strongly inhibited by 8-azaguaninne (9). We were worried, however, that the 8-azaguaninne appeared to be more generally toxic but our later studies showed that antibody formation was selectively inhibited by inhibitors of DNA synthesis while the synthesis of other proteins was less affected, suggesting that antibody synthesis was somehow dependent on DNA synthesis (10). We were able to confirm that the antibody forming cells were dividing (11) using an early version of the hot pulse technique and, in 1962, we showed that antigen actually stimulated DNA synthesis as measured by the increased uptake of tritiated thymidine in cultures of lymphocytes from previously immunized rabbits (12). It is, perhaps hard to believe from our current perspective, that this was a novel, exciting finding, but the "obvious" is often not "obvious" until it is "obvious."

At that time, we did not know about T-cells and B-cells and the role of T-cells in the B-cell response, and we assumed that the dividing cells were the antibody forming cells. Now, we would presume that T-cells are also a component and the assay soon became a major assay in the hands of Benacceraf, and others, for many T-cell studies (13).

Later, in 1966, Mishell and I developed a more sophisticated *in vitro* model (14) in which we could generate a primary antibody response of mouse spleen cells to various erythrocyte antigens and it was this that we used to show that responses to different antigens were carried out by different cells. By this time, we had adopted the use of the hemolytic plaque assay (15), developed by Jerne and Nordin (16), and we could measure the actual number of cells making antibodies to erythrocyte antigens. We started the response to antigen A, killed the cells making the response by letting the dividing cells incorporate highly radioactive tritiated thymidine, diluted the tritiated thymidine with unlabeled thymidine, and then started the response to antigen B. The two antigens were sheep erythrocytes and burro (donkey) erythrocytes, and the results were the same whether we started with burro or sheep. Other experiments in the paper showed that the first round of proliferation did not begin until 24 h after the addition of antigen to naïve cells but was earlier if the cells were from immunized donors and that virtually all the antibody forming cells arose from the extensive proliferation of a much smaller number of precursor cells.

The idea for the hot pulse technique, which we used here and in our earlier paper (11), came from my undergraduate days where I had learned of the studies of Hershey et al. (17) in which they showed that phage infection of *Escherichia coli* could be progressively destroyed if the DNA was labeled with radioactive 32P. In their technique, it was the disintegration of the 32P that destroyed the link between successive nucleotide triphosphates of the DNA, while in our technique (11), we showed that it was the very soft beta irradiation of the incorporated tritium that killed only the cells that had incorporated the hot thymidine as they synthesized new DNA.

Subsequent studies with the same *in vitro* system led to the identification of a T-cell replacing factor (18), the effect of mitogens on T-cell help (19), positive and negative allogeneic effects (20), and the true nature of the relationship between CD8 and CD4 T-cells and the recognition of Class I and Class II MHC (21, 22).

In my memory, I had seen us led inexorably to the truth by a series of searing insights but as I reread the papers I see that we only stumbled our way to a better understanding.

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The story behind "a requirement for two cell types for antibody formation *in vitro*"

Donald E. Mosier*

Department of Immunology and Microbial Science, The Scripps Research Institute, La Jolla, CA, USA
**Correspondence: dmosier@scripps.edu*

Edited by:
Kendall A. Smith, Weill Cornell Medical College, USA

Reviewed by:
Christopher E. Rudd, University of Cambridge, UK
Harry W. Schroeder, University of Alabama at Birmingham, USA

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This is a personal story about how to get your scientific career started with a bang. It may not be as easy today as it was in the 1960s, but I think the principles still apply. I hope that this personal recounting will be useful to young scientists trying to establish their career.

GET INTO A GOOD LABORATORY AS SOON AS YOU CAN

The way to get your scientific career started is to work with great scientists who are working on important problems in an exciting environment. I spent the summer of 1964 working as a technician in the laboratory of Brigitta Askonas at the National Institute for Medical Research (NIMR) in Mill Hill, a northwest suburb of London. Askonas was a rigorous scientist who was interested in the role of macrophages in the antibody response (1). The job had been arranged by Charles Medawar, a friend during my undergraduate days at Indiana University (IU), and son of Sir Peter Medawar, who was then the Director of the NIMR and a Nobel Laureate for the discovery of immune tolerance. It helped that I had worked in the laboratory of Felix Haurowitz at IU studying rabbit antibody responses to closely related haptens, and thus had some introduction to the field of immunology, and that Professor Haurowitz was friends with Sir Peter. So before I arrived at the University of Chicago to begin medical school in the fall of 1965, I had spent time with three prominent immunologists and I had some sense of the critical issues in the field and felt comfortable at the lab bench. This was just after thymus-derived (T) cells and bone marrow-derived (B) cells had been identified (2), and the cellular requirements for antibody formation were still unknown.

FIND GOOD MENTORS AND DO NOT GET DISCOURAGED IF AT FIRST YOU DO NOT SUCCEED

My first year in medical school was a disappointment. I liked the excitement of a research lab, not the rote learning of anatomy, physiology, and biochemistry. During the spring of 1966, I remember attending a lecture by Don Rowley and being impressed by his enthusiasm and energy. I decided to take a leave of absence from medical school to pursue a Ph.D. (this was prior to the combined M.D./Ph.D. training program). Since Don Rowley and Frank Fitch were in the Department of Pathology, that was my first choice of graduate department. Bob Wissler was then chair of the department and I was assigned by him to a new junior faculty member that left me with lots of lab space and little mentoring. I attempted, at first on my own, to follow up on two seminal technical publications that had appeared in 1963 and 1966. The first was the report by Neils Jerne and Al Nordin (3) that described a method for detecting single antibody-producing cells by a hemolytic plaque assay using sheep red blood cells (SRBC) as the antigen. The second was the report by Bob Mishell and Dick Dutton (4) describing *in vitro* antibody formation against SRBC in mouse spleen cell cultures. My lab notebook from 1966 records many attempts to perfect the Jerne plaque assay, at least some of which were successful, and more attempts to perfect the Mishell–Dutton technique, none of which were successful. I was spending more time talking with Rowley and Fitch trying to solve these problems. They were an ideal team of mentors, with Rowley constantly getting excited about the hypothesis of the day, and Frank Fitch gently guiding the experimental details in his gentlemanly fashion. In January of 1967, they proposed the brilliant idea of sending me to Dutton's lab at the Research Institute of Scripps Clinic (now renamed as The Scripps Research Institute, where I have been since 1992) in La Jolla, CA, USA. A trip from midwinter Chicago to sunny La Jolla seemed like a great idea to me. Bob Mishell and Dick Dutton were very cordial hosts (Dick Dutton is a lifelong friend), and I learned all the secrets of the culture technique that were not revealed in their paper, such as, only selected sources of SRBC and fetal calf serum would work, the rabbit complement for developing the hemolytic plaques needed to come from the right rabbit, and adding new medium to the cultures each day was essential. They also showed me their plastic culture boxes that were much easier to use for the required gas mixture than the desiccator jars that I had been using in Chicago (I still have several of these culture boxes in my lab these many years later). I was able to take this information back to Chicago and finally achieve success by mid-March of 1967, with literally hundreds of plaque-forming cells (PFC) per million mouse spleen cells cultured.

ASK IMPORTANT QUESTIONS

One of the critical questions in immunology was the role of "macrophages" in the antibody response. Several papers had suggested that incubation of macrophages with antigen enhanced the immune response when the mixture was injected into animals, but their role in promoting antibody formation was unknown at the

time. There were some crazy (in retrospect) theories floating around, like RNA from macrophages instructing B cells to make antibody (better not cited). This was long before we knew how antibody variability was generated, and instructional theories of antibody folding favored by Felix Haurowitz still had many proponents. The new Mishell–Dutton culture technique provided the opportunity to address the role of macrophages *in vitro* since inspection of the tissue culture dishes by phase microscopy (I was lucky to be in a pathology department) clearly showed large, adherent phagocytic cells that were often surrounded by loosely or non-adherent mouse splenic lymphocytes[1]. It was known at the time that macrophages would adhere to glass (5), but adherence to plastic culture dishes was a novel observation. My lab notebook shows several experiments where spleen cells were separated into "sticky" and "non-sticky" cells, then later revised to "adherent" and "non-adherent" cells, and a final nomenclature evolution to "macrophage-rich" and "lymphocyte-rich" during the preparation of my 1967 manuscript (6). It was apparent by late April of 1967 that neither "macrophage-rich" or "lymphocyte-rich" spleen cell subpopulations alone could respond to SRBC, but adding SRBC to the "macrophage-rich" subpopulation for 30 min prior to recombining with the "lymphocyte-rich" subpopulation allowed a robust PFC response that was comparable to intact spleen cells. Experiments were numbered sequentially in my notebook, and the productive replicate experiments in April to June 1967 were numbered 29–36, each involving 4–5 days of culture before the Jerne PFC assays. For young scientists starting out, it is worth noting that most of the first 25 experiments performed over a period of 8 months were not productive, so be patient, seek help, and collaborate with experts.

ACCEPT HELP GRACIOUSLY

Speaking of large numbers of experiments, there were also large numbers of manuscript drafts over the summer of 1967 before the final version was submitted to Science in October. This was my first paper, and I spent many hours with Don Rowley refining each word in the manuscript, shortening it to reach the word limit for Science, hand drawing Figures 1 and 2 in India ink (I still have the faded original Figure 1 in my notebook), and refining the technical notes that would now appear as supplemental information but were then included in the references. Frank Fitch also reviewed the manuscript, but it was Don Rowley who insisted that it meets his high standards – the 30th revision finally made the cut. After all this effort, I was amazed that both Don Rowley and Frank Fitch insisted that they should not be co-authors. This was generous by the standards of the day, and would be next to impossible in the current climate of underfunded scientists scrambling for NIH grants.

Subsequent work that I performed in Don Rowley's laboratory suggested that the cell required for the SRBC antibody response in the "macrophage-rich" subpopulation was quite rare (7) and unlikely to be the predominant macrophage. This led to a reversion of the nomenclature to "adherent" cell, and later work by Ralph Steinman identified the key adherent cell to be the dendritic cell. Once again, Rowley and Fitch declined to be co-authors on my paper, but the mathematician who was familiar with limiting dilution analysis, Lionel Coppleson, was appropriately acknowledged. In 1969, I was convinced to return to medical school, but I continued to work part-time in the Rowley lab with fellow students Jeffrey Roseman, Lee Leserman, and Bob Waterston. Great times in a great laboratory! When I finished medical school, I went back to Mill Hill for a short but intense post-doc training with Avrion Mitchison, where I met Martin Raff and Harvey Cantor, and enjoyed many hours of discussion and a few hours at the bench.

In retrospect, my first paper was one of my best, and helped establish the concept that antibody production by B cells was dependent upon cell cooperation between antigen-presenting cells and the responding lymphocytes. Subsequent work (7, 8) showed that T helper cells were also required for the antibody response, with the last paper finally including Frank Fitch and Don Rowley[2] as co-authors. This requirement for three cell types had been predicted by the limiting dilution experiments (7), and led to much subsequent work by many immunologists on antigen-processing, helper T cell function, and the genetic basis for antigen recognition by T cells and B cells.

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[1]Rowley and Fitch had been studying antibody responses in rats *in vivo*, but we were never able to adopt the Mishell–Dutton technique to work with rat spleen cells in culture.

[2]Sadly, Donald Adams Rowley died in February of 2013, and I was one of many of his former students who gathered in Chicago to pay tribute to him last spring. This article is dedicated to him, a generous and skilled mentor whose contribution to the start of my career was unique and is appreciated more with each passing year.

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GENERAL COMMENTARY
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Evolution of the serendipitous discovery of macrophage–lymphocyte interactions

Joost J. Oppenheim*

Laboratory of Molecular Immunology, National Cancer Institute, National Institutes of Health, Frederick, MD, USA
**Correspondence: oppenhej@mail.nih.gov*

Edited by:
Kendall A. Smith, Weill Medical College of Cornell University, USA

Reviewed by:
Pietro Ghezzi, Brighton and Sussex Medical School, UK
Irina Caminschi, Burnet Institute, Australia

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A commentary on

The transformation of column-purified lymphocytes with nonspecific and specific antigenic stimuli
by Oppenheim JJ, Leventhal BG, Hersh EM. J Immunol (1968) 101:262–70.

This contribution to the project on the Living History of Immunology concerning "The Transformation of Column-Purified Lymphocytes with Non-specific and Specific Antigenic Stimuli," by Joost J. Oppenheim, Brigid G. Leventhal, and Evan M. Hersh represents another excellent example of research based on a serendipitous discovery snatched from the jaws of a failed project (1). Evan, Brigid, and I were all clinical associates at the NCI engaged in the care of patients with leukemia and solid tumors from 1962 until 1965. As a reward for our clinical efforts, we were given the opportunity to pursue laboratory research studies with one of the principal investigators for the last 2 years of our stay.

Following several false starts, I ended up in the laboratory of Dr. Jacqueline Wang Peng, who was an expert in studies of chromosome abnormalities caused by neoplastic changes and damage from chemotherapeutic and radiation treatments. Our chromosome analyses were frustrated by the failure of leukemic lymphocytes from chronic lymphocytic leukemia (CLL) patients to be activated to divide and develop metaphases that could be analyzed for chromosome breaks in response to a kidney bean extract known as phytohemagglutinin (PHA). Since the peripheral blood (PB) of more advanced CLL patients contained high numbers of white blood cells (WBC) consisting entirely of lymphocytes, we decided as a control to purify the non-adherent lymphocytes present in normal PBWBC by eluting them off sterile glass bead or nylon fiber columns, which retained the adherent phagocytic neutrophils and monocytes.

After numerous mishaps and considerable practice, these columns yielded at least 98% pure lymphocytes based on microscopic analysis. We were dismayed to find that these purified normal lymphocytes were also hyporesponsive to a variety of antigenic stimulants such as tetanus toxoid and streptolysin O, but still showed normal proliferative response to the more potent polyclonal PHA stimulant. This was determined from the proportion of cells undergoing morphological blastogenesis and the uptake of tritiated thymidine. However, the purified lymphocytes in comparison with unpurified normal lymphocytes were also hyporesponsive to suboptimal doses of PHA. Of course, we were very concerned that the column procedure had damaged the cells, but we failed to observe any evidence of cell death based on trypan blue uptake. Furthermore, when cultured at a higher cell density, the lymphoproliferative response to antigens showed some recovery arguing against cell damage. This observation also suggested the possibility that the few residual contaminating non-lymphocytic cells might be interacting more effectively over the shorter distances at higher cell densities. We tested this idea by adding some unfractionated WBC back to the cultures of purified lymphocytes, which partially restored the lymphoproliferative response to antigens. A feeder layer consisting of WI-38 human embryonic fibroblasts had no restorative effect.

These results unfortunately failed to shed any light on the unresponsiveness of CLL cells. However, they pointed to the requirement for a cooperative interaction between phagocytic cells and lymphocytes. Based on the available literature, we proposed that macrophages were somehow facilitating the activation of lymphocytes to "transform" and proliferate. Evan Hersh and Jules Harris obtained convincing evidence in support of this hypothesis by restoring the lymphoproliferative responses by the addition of coverslips with adherent human macrophages to the cultures of purified lymphocytes (2).

I further pursued my immunological studies during a sabbatical year at the University of Birmingham in England from 1965 to 1966, where I learned to work with non-human species and showed that purified lymphocytes from guinea pig lymph nodes were also unresponsive to antigenic stimulants unless supplemented with some phagocytic cells. Upon returning to the Dental Institute at the National Institutes of Health (NIH), I was joined by a pediatrician, Dr. Robert Seeger in investigations of the role of macrophages in immunity. We were able to show that footpad injection of peritoneal macrophages from syngeneic guinea pigs after a brief exposure to antigens such as ovalbumin induced greater delayed hypersensitivity (DTH) reactions and were better at priming antibody responses than equal or higher doses of soluble antigens (3). Furthermore, antigens were taken up much less well by lymphocytes, thymocytes, and hepatoma cells than to macrophages and these cells were

not immunogenic (4). Thus, macrophages could activate T lymphocytes to mediate DTH and prime B-cell antibody production. Bob Seeger and I also determined that peritoneal, alveolar, or PB macrophages obtained from either immune or non-immune donors were all equally effective at priming immune responses (5). However, macrophages could not induce non-immune lymphocytes to proliferate. Thus immune specificity and memory appeared to be a property of lymphocytes rather than macrophages.

In the course of our studies, Bob and I noticed that syngeneic macrophages were much more effective than allogeneic macrophages, but we did not pursue this issue. Alan Rosenthal thoroughly investigated the role of histocompatibility in this interaction. Alan Rosenthal and Ethan Shevach went on to show that the macrophage–lymphocyte interactions required MHC compatibility to be successful (6). They further determined, using alloantisera against MHC antigens, that macrophage MHC was necessary for T lymphocytic recognition of antigens (7). Of course, Ralph Steinman's discovery that dendritic cells (DC) contaminating the macrophage preparations were actually the most potent antigen presenting cells superseded our findings (8). However, I must confess that I found it difficult to accept the idea that the small contaminant population of DC rather than macrophages was responsible for antigen presentation, until this became incontrovertible based on the *in vitro* studies of Jacques Banchereau and his colleagues (9). They were able to produce large numbers of dendritic/Langerhans cells *in vitro* by culturing cord blood hematopoietic progenitor cells with a combination of granulocyte-macrophage colony stimulating factor and tumor necrosis factor. These cells had the morphology and phenotypic markers of DC and were very potent at presenting antigens and priming T lymphocytes.

Our serendipitous finding that T cells require accessory cells for antigen presentation was based on an initial desire to better understand the failure of lymphocytes from CLL patients to transform in response to stimulation. Other investigators have determined that CLL cells are usually monoclonal B lymphocytes that do not respond to T cell stimulants (10). This was followed by curiosity on our part to better understand the inability of purified normal peripheral human lymphocytes to respond to antigenic stimulation unless supplemented by macrophages. Our observations led other investigators to discover the crucial role of MHC in antigen presentation, antigen processing, and the outstanding capacity of DC to activate T cell-dependent immune responses. These consequent findings went beyond the scope of our imagination. In conclusion, our unexpected scientific findings clearly contributed in an unanticipated manner to a greater understanding of adaptive immunity and clearly illustrate the stepwise communal process of scientific progress.

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Defining cell-surface antigenic markers for mouse T and B cells

Martin C. Raff*

MRC Laboratory for Molecular Cell Biology, University College London, London, UK
**Correspondence: m.raff@ucl.ac.uk*

Edited by:
Kendall A. Smith, Weill Medical College of Cornell University, USA

Reviewed by:
Bruno Laugel, Cardiff University School of Medicine, UK
Michael Loran Dustin, Harvard University, USA

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A commentary on

Theta isoantigen as a marker of thymus-derived lymphocytes in mice
by Raff MC. Nature (1969) ***224****:378–9.*

Two distinct populations of peripheral lymphocytes in mice distinguishable by immunofluorescence
by Raff MC. Immunology (1970) ***19****:637–50.*

I began my scientific career in October 1968 at the National Institute for Medical Research (NIMR) in London. I was 30-years old, having just finished my training in clinical neurology in Boston, and I had come to work with Avrion (Av) Mitchison. I had much to learn, as I had done no basic research and knew very little about immunology.

It was an exciting time – both in immunology and at the NIMR. There was increasing evidence that there were two types of lymphocytes, T and B cells, responsible for adaptive immune responses but there were no good ways to distinguish or separate them. Av had recently heard the Boston immunologist Arnold Reif describe a mouse isoantigen called theta (θ), which was present in the brain and on the surface of thymocytes (1, 2). Av wondered if θ might be present on T but not B cells, in which case it could serve as a useful cell-surface marker for mouse T cells. He gave me the relevant Reif papers, an aliquot of a mouse anti-θ antiserum that he had begun making, and set me free.

In a commentary written in 2008 for the Pillars of Immunology series in the *Journal of Immunology* (3), the Seattle immunologist Pamela Fink colorfully described what happened next. Here, I give an abbreviated account of this history but broaden it to include the fortuitous finding that immunoglobulin (Ig) can serve as a cell-surface marker for B cells.

To detect θ on mouse lymphocytes, I first used an antibody- and complement-dependent ^{51}chromium-release cytotoxicity assay, which I learned from my lab-mate Marion Ruskowicz (4). I found that the antiserum and complement killed essentially all thymus lymphocytes but only a subset of lymph node and spleen lymphocytes. To test whether the θ-positive lymphocytes in lymph node and spleen were T cells, I analyzed cells from pathogen-free mice that had been treated since birth with a rabbit antiserum made against mouse thymocytes (5) and were therefore T-cell-depleted; Sandra Nehlson, a Ph.D. student with Peter Medawar who worked across the hall, generously provided these mice. I found that the spleen and lymph nodes of the mice contained normal numbers of θ-negative lymphocytes but greatly reduced numbers of θ-positive lymphocytes, strongly suggesting that Av's hunch was right – θ is present on T but not B cells (6). Schlesinger and Yron independently published very similar findings around the same time (7); unfairly, their paper received far less attention, probably because its title lacked the punch line of their findings. Later, in collaboration with Henry Wortis, who worked next door, we confirmed these findings in other T-cell-deficient mice, including congenitally athymic nude mice (8).

I then tested the functional properties of θ-positive spleen cells by analyzing the cells involved in an adoptive cell-transfer system that Av had developed to study the cooperation between two populations of spleen cells – one from mice immunized with a hapten (NIP) coupled to a carrier protein (chicken γ-globulin, CGG) and another from mice immunized with an uncoupled, second carrier protein (bovine serum albumin, BSA). He had shown that, when both cell populations, but not either one alone, are transferred into a sub-lethally irradiated mouse, the recipient mouse produces large amounts of anti-NIP antibodies in the blood when immunized with NIP-BSA but not with NIP-CGG – an example of the so-called carrier effect (9). In my experiments, before transferring the cells, I treated one or other population with anti-θ antibodies and complement to kill the T cells, using normal mouse serum plus complement as a control. In this way, I could show that the relevant cells in the BSA-immunized population were T cells, whereas the relevant cells in the NIP-CGG-immunized population, which produced the anti-NIP antibodies (9), were not (10). This experiment provided direct evidence that T cells recognizing antigenic determinants on a protein can help B cells make antibodies against different antigenic determinants on the same protein (11). It also established the value of antibodies that recognize cell-type-specific surface antigens, which rapidly became standard tools in immunology and, later, in various other branches of biology.

Remarkably, Av declined to put his name on these two *Nature* papers (6, 10), even though the projects were his idea and he had begun to produce anti-θ antibodies before I arrived in London. This exceptional generosity had an enormous influence on my career. Theta (now called Thy-1) rapidly became a standard marker for mouse T cells, and the two single-author

Nature papers gave me immediate international recognition, after only 2 years doing basic science. Nonetheless, if I had known then what I know now, I would have insisted that Av's name was on the papers, to indicate his crucial contributions to the work. Av always did his own experiments and made many landmark contributions to immunology; because he usually allowed his students and postdoctoral fellows to publish on their own, however, his actual contributions are far greater than are documented in the literature.

To visualize θ directly on the surface of living T cells, I turned from cytotoxicity assays to immunofluorescence experiments. In these experiments, I visualized the bound mouse anti-θ antibodies using fluorescent rabbit anti-mouse-Ig antibodies. A surprise came from control experiments in which I omitted the anti-θ antibodies and found that the fluorescent anti-Ig antibodies on their own labeled a substantial proportion of lymphocytes in cell suspensions prepared from various peripheral lymphoid organs, although not in suspensions of thymocytes. Roger Taylor, working across the hall with Michel Sternberg, had independently obtained similar results using radiolabeled anti-mouse-Ig antibodies, and we published our findings together (12). Although a number of immunologists, including Av, had suspected that the antigen receptors on lymphocytes might be Ig proteins, ours was one of the first direct demonstrations of Ig molecules on the surface of lymphocytes.

The finding of Ig on some peripheral lymphocytes but not others raised the question of which class of lymphocyte expressed the cell-surface Ig. To find out, I studied lymphocytes from normal mice and from various T-cell-depleted mice, labeling the cells with anti-mouse-Ig antibodies, with or without first labeling them with mouse anti-θ antibodies. The results were unambiguous: the Ig-positive cells were θ-negative, implying that they were B cells, whereas the θ-positive T cells were Ig-negative (13). (The analysis was greatly helped by the fact that the Ig was distributed in a cap at one pole of the B cells, whereas θ was distributed as a ring on the T cells; later, Stefanello de Petris and I, and Roger Taylor and Phillip Duffus independently, showed that the binding of the anti-Ig antibodies induces the B cells to actively redistribute their surface Ig molecules into a cap (14) – but that is another story.) The *Journal of Experimental Medicine* rejected my paper as not being sufficiently interesting, and it was published in *Immunology*, a low impact journal. Despite this (and its unhelpful title), it became a Citation Classic (15), which taught me an important lesson: it is what you publish rather than where you publish it that matters most. The finding of Ig on the surface of B cells but not T cells led to a prolonged and frustrating search by many laboratories for the antigen receptors on T cells, which were only identified as distinct Ig-like proteins years later, after a number of false leads (16).

Cell-surface Ig became a standard marker for B cells in all vertebrates. When I moved with Av to University College London, for example, John Owen and I collaborated with Max Cooper (who was on sabbatical from the University of Alabama) and used anti-Ig antibodies and explant cultures to study the development of B cells. We showed that mouse B cells develop in the fetal liver and adult bone marrow (17), rather than in gut-associated lymphoid tissues as had been proposed by Max and others. We later used Max's class-specific anti-Ig antibodies to demonstrate that the B cells arise from pre-B cells, which have already begun to make IgM heavy chains (18).

Remarkably, these first few years in science were the most productive in my research career. This early success was largely the result of good luck: I was at the right place at the right time, with a generous and inspiring mentor. And it was why I became a scientist rather than a practicing neurologist.

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The discovery of T cell–B cell cooperation

N. Avrion Mitchison*

University College London, London, UK
**Correspondence: n.mitchison@ucl.ac.uk*

Edited by:
Kendall A. Smith, Weill Medical College of Cornell University, USA

Reviewed by:
Ellis L. Reinherz, Dana-Farber Cancer Institute, USA

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A commentary on

The carrier effect in the secondary response to hapten-protein conjugates. II. cellular cooperation
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The carrier effect in the secondary response to hapten-protein conjugates. I. Measurement of the effect with transferred cells and objections to the local environment hypothesis
*by Mitchison NA. Eur J Immunol (1971) **1**: 10–7. doi:10.1002/eji.1830010204*

Until the mid-twentieth century, immunology had been very much a matter of soluble antibodies and their effect on the antigens of bacteria and viruses. Then, in the wartime and post-war years, a new area opened, of cell-mediated immunity, driven initially by interest in the ubiquitous rejection of homografts in man and animals. Experimental tolerance was a key discovery, that introducing donor-type cells before the ability to reject homografts had developed could prevent the rejection. Hašek in Czechoslovakia made the discovery independently in 1953 and by Billingham, Brent, and Medawar in Britain in 1954.

Proof that rejection of homografts is immunological in nature came from the discovery by the Medawar group that skin grafts are rejected more rapidly if the host has already rejected previous grafts from the same donor. My contribution was to show that this accelerated reaction could be transferred from one inbred mouse to another by means of spleen cells (1), work that I later continued in the laboratory of George Snell at Bar Harbor, ME (2).

Returning to UK, and after a period in Edinburgh University, I joined the National Institute of Medical Research, where Medawar had become director. My experience in Edinburgh with chicken erythrocytes had taught me the value of radioactive labeling (3), so I sought to adapt this technology (fairly new at the time) to tracking serum antibody levels in the small blood samples available in mouse studies. Down the passage worked Rosalind Pitt-Rivers, discoverer of the thyroid hormone T3, a great friend. Jointly we designed NIP-CAP, a structure related to T3 that can (i) serve as a powerful hapten because of its nitro and hydroxyl groups, (ii) couple smoothly to proteins to form part of immunogenic molecule, and (iii) can be prepared in radioactive form at the iodine residue and thus be used to assay binding of NI^{131}P-CAP to its antibody (4). Together these properties opened the way to an easy mouse serology; indeed for a while, it became so widely used that the *European Journal of Immunology* accepted its name as not requiring further explanation.

My work focused on an aspect of immunological memory, the carrier effect. An individual primed by injection of a hapten–protein conjugate makes a full secondary anti-hapten antibody response only to the same conjugate, but not to the same hapten conjugated to another protein. This finding suggested to us that two cells might be involved, one recognizing the hapten and the other the carrier protein. To explore this possibility, we devised a serology applicable in mice (5). The small samples of serum available were appropriately diluted and then incubated with 10^{-8} M NI^{131}P-CAP; their immunoglobulin was then precipitated by addition of ammonium sulfate solution and centrifuged, carrying the bound radioactive hapten down with it. By this method, anti-NIP antibody could be detected down to a concentration of $\sim 10^{-9}$ M, as available with adoptively transferred spleen cells. This transfer system could then be used to explore the carrier effect as defined above. The secondary response obtained from the transferred spleen cells was indeed much reduced (~1000-fold) when the cells were stimulated with the same hapten (NIP) attached to a different carrier protein such as bovine serum albumin, compared to stimulation with the NIP-chicken γ-globulin originally used to immunize the cell donor. Importantly, the transferred anti-NIP response could be inhibited by injecting an excess of carrier protein, indicating that the carrier protein was itself recognized independently of the hapten that it carried, and thus that a second population of reactive cells was involved independent of those that recognized the hapten.

Our experimental design took spleen cells from mice immunized with NIP-OA (NIP conjugated with ovalbumin) plus adjuvant and transferred them into irradiated host mice that were then challenged with either NIP-BSA (NIP conjugated to bovine serum albumin) or NIP-OA (NIP conjugated to ovalbumin), both without adjuvant. The molar concentration anti-NIP antibody made in response was then measured, and its level titrated against the quantity of antigen in the challenge. Typically, mice needed a higher dose of the heterologous antigen (NIP-BSA) than of the homologous one (NIP-OA) to achieve the same level of anti-NIP antibody. Adding spleen cells from mice immunized with BSA alone to the

transferred cell population increased sensitivity to NIP-BSA 10–100-fold, a finding that defines the "carrier effect." The effect is specific, as the increase was not obtained with spleen cells from mice immunized with HSA (human serum albumin). These BSA-primed cells did not contribute directly to the anti-NIP antibody, as judged by allotype markers on the antibody; they acted only as "helper cells."

Thus, these findings reveal a carrier effect mediated by the immune system, but not by antibody. To test for a T cell-mediated effect, cells were obtained from the spleen of mice that 7 days earlier had been irradiated and then reconstituted intravenously with 90×10^6 syngeneic thymus cells and immunized with BSA, alum, and pertussis (6). These cells were tested for helper activity by transfer into irradiated syngeneic hosts, along with the usual NIP-BSA as immunogen. The transfer significantly increased the host anti-NIP antibody response, in proportion to the number of BSA-primed cells transferred.

Such experiments became easier later, when T cells could be manipulated by means of anti-theta antibody [reviewed by Raff (7, 8)]. By then, it had become clear that cooperation between T and B cells as revealed by the 1971 study considered here, most likely works through an antigen bridge between epitope-specific receptors on both cells. B cells, with their immunoglobulin receptors, recognizing the matrix of epitopes presented on the surface of T cells, became and remain the accepted mechanism of T–B cooperation in the immune response.

T cells and their interactions with other cells have become a major theme in immunology. Th interactions lie at the heart of inflammation and other aspects of immunological and infectious disease, and are increasingly being manipulated via monoclonal antibodies directed at cell surface markers and via cytokines. Advances in the molecular biology of the cell underpin these developments. These are extremely active fields, with much to offer in molecular cell biology and via therapeutic intervention.

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The definition of lymphocyte activating factor: giving a Helping Hand to Serendipity

Igal Gery*

Laboratory of Immunology, National Eye Institute, National Institutes of Health, Bethesda, MD, USA
**Correspondence: geryi@nei.nih.gov*

Edited by:
Kendall A. Smith, Weill Medical College of Cornell University, USA

Reviewed by:
Kendall A. Smith, Weill Medical College of Cornell University, USA
Pietro Ghezzi, Brighton and Sussex Medical School, UK

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A commentary on

Potentiation of the T-lymphocyte response to mitogens. I. The responding cell
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Potentiation of the T-lymphocyte response to mitogens. II. The cellular source of potentiating mediator(s)
by Gery I, Waksman BH. J Exp Med (1972) 136(1):143–55. doi: 10.1084/jem.136.1.143

The idea that soluble cell products play important roles in the complex process of the immune response was supported by scientific evidence in the sixties in several publications including those by David et al. (1) and Bloom and Bennett (2), who discovered the activity of macrophage migration inhibitory factor (MIF), Gordon and McLean (3) who reported that media of leukocyte cultures contain mitogenic factors, Kasakura and Lowenstein (4), who showed the activity of a "blastogenic factor" and Ruddle and Waksman (5), who discovered the activity of lymphotoxin. Further, a 1970 paper by Bach et al. (6) reported on a soluble factor that could replace macrophages in purified lymphocyte proliferative responses.

Unlike the factors mentioned above, the definition of lymphocyte activating factor (LAF) was a result of a study aimed at an entirely different purpose. In May 1970, I came to the lab of Byron Waksman, at Yale University, for a sabbatical from my position at the Hebrew University Medical School. Prior to coming to Yale I acquired expertise in culturing lymphocytes and Richard (Dick) Gershon, who collaborated with Waksman's group, approached me with the idea to use this expertise for studying the activity of the "suppressor cells" he discovered. It is of note that the concept of a population of lymphocytes whose function is suppression of immune responses by other lymphocyte populations was revolutionary at that time and Dick had to struggle to get his data published; his seminal paper on suppressor cells was finally accepted by *Immunology* (7). The experiment Dick suggested was to inject naïve mice with large ("tolerizing") doses of sheep red blood cells (RBC) and to test lymphocytes from these treated mice for their proliferative responses in culture to the mitogen phytohemagglutinin (PHA) in the presence of sheep RBC. We focused on spleen lymphocytes of the treated mice, but we also examined the response of thymus cells of the injected mice. For specificity controls we used human whole blood cells (from a colleague donor). The mouse spleen cultures exhibited a moderate level of suppressed response to PHA (8). In addition, however, I noticed an unexpected response: vigorous proliferation by thymus cell cultures incubated with human RBC (our control. . .) and PHA. The thymocytes did not respond, however, to PHA alone. Further analysis of the unexpected finding established that this was not a fluke and revealed that the thymocyte response was actually triggered by the small number of leukocytes that "contaminated" the fresh human RBC preparation. Indeed, when using purified human blood leukocytes, as few as 6,000 of these cells, along with PHA, were sufficient to stimulate a significant response by the thymocytes. The next step was, obviously, to examine the supernatant of human leukocytes for stimulatory activity and I was delighted to find that the supernatant had strong stimulatory activity on the thymocyte response to PHA. We believed that these preliminary observations were of sufficient importance and summarized them as a short communication in the *Journal of Immunology* (9).

During the following months of my sabbatical at Yale I expanded the research, focusing on two related issues: the analysis of the responding cells and of the cells that produce the stimulating factor. At that stage we had to give the factor a name and we chose "Lymphocyte activating factor" or "LAF". The best responding cells were identified to be the more mature thymocytes, but LAF also stimulated the response of less mature thymocytes, as well as the response to stimulants by murine spleen cells. Useful information was particularly provided by the analysis of the LAF producing cells and their stimulants. High levels of LAF activity were secreted by adherent cells (mostly macrophages) stimulated by lipopolysaccharide (LPS) and by non-adherent lymphocytes stimulated with PHA or concanavalin A. In hindsight, it seems that the macrophages produced LAF activity, whereas the PHA-stimulated lymphocytes released mainly another stimulatory factor. It is also noteworthy that the LPS used in these preliminary experiments was a gift from the lab of Elisha Atkins, two floors below our lab. Elisha and his group had been using LPS as a stimulant for the production of the endogenous pyrogenic factor they measured by inducing

fever in animals. That batch of LPS was also used in a study we carried out at the same time showing for the first time that LPS is mitogenic for B-cells (10).

Before leaving New Haven I left with Byron Waksman drafts for two manuscripts that summarized the data. Byron rewrote the manuscripts that were accepted for publication by the *Journal of Experimental Medicine*. The first paper (11) is co-authored by Byron Waksman and Dick Gershon, but Dick was not included in the authors' list of the second one (12). This was clearly unfair to Dick and I certainly feel badly about it.

During my last months in New Haven I also contacted Bob Handschumacher, at the Department of Pharmacology at Yale, to help me with the characterization of LAF. We carried out some preliminary experiments, but in order to complete the study I returned to New Haven in the summer of 1972 and together with Bob we collected some basic information on the factor, including the finding that LAF is a protein, with a size of ~15 kDa. We submitted a manuscript that summarized the data to the *Journal of Immunology*, but the manuscript was rejected. We then submitted it to *Cellular Immunology* (13) and were delighted to learn that this paper was subsequently highly cited.

Later studies by other groups [e.g., Ref. (14)] revealed that highly purified "LAF" preparations functioned to promote the production of a T cell-derived activity, which was the basis for the interleukin nomenclature, first proposed at the Second International Lymphokine Workshop in 1979 at Ermatigen Switzerland. Thus, "interleukin-1 (IL-1)" made by macrophages was distinguishable from "IL-2", made by lymphocytes (14).

Looking back, I feel lucky for including the human "RBC" control cultures in the initial experiment performed 44 years ago and proud for pursuing the unexpected and weird result by the subsequent experiments that yielded the definition of the LAF.

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GENERAL COMMENTARY
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Demonstration of functional heterogeneity of T lymphocytes and identification of their two major subsets

Paweł Kisielow*

Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Wrocław, Poland
**Correspondence: kisielow@iitd.pan.wroc.pl*

Edited by:
Kendall A. Smith, Weill Medical College of Cornell University, USA

Reviewed by:
Christopher E. Rudd, University of Cambridge, UK
Kendall A. Smith, Weill Medical College of Cornell University, USA

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A commentary on

Ly antigens as markers for functionally distinct subpopulations of thymus-derived lymphocytes of the mouse
by Kisielow P, Hirst JA, Shiku H, Beverley PCL, Hoffmann MK, Boyse EA, et al. Nature (1975) 253:219–20. doi: 10.1038/253219a0

Cells responsible for the specificity of the immune response and thymus function remained unknown until mid 1960s when lymphocytes were identified as mediators of cellular and humoral immunity (1–4). Soon afterward *in vivo* reconstitution experiments showed that cellular and humoral immunity are mediated by two different types of lymphocytes: thymus-derived T cells and thymus-independent, bone marrow derived antibody producing B cells, respectively (5). This finding created a need for a simple and rapid method to distinguish these two classes of lymphocytes, and the immune system itself provided the best tool for this task. In 1969, Martin Raff (6) showed that antibodies to Thy1 antigen [cell surface molecule expressed chiefly in the thymus and brain (7)] react with all thymus-dependent but not with thymus-independent lymphocytes. This allowed the distinguishing and separation of functionally different but morphologically identical lymphocytes allowing study of their specific activities. Meanwhile Edward Boyse, Lloyd Old, and colleagues identified two other cell surface antigens expressed mainly in the mouse thymus, Ly1 and Ly2 (8), which were thought to characterize all T cells, like Thy1.

By that time T cells were known to have several functions and an important question emerged: do different functions reflect their functional heterogeneity or are manifestations of homogenous population of multifunctional cells?

Late in 1972, I obtained a postdoctoral WHO fellowship. Being interested in lymphocyte biology and cell surface immunogenetics I contacted Dr. Lloyd Old, who thanks to the recommendation of my PhD supervisor, Professor Czesław Radzikowski invited me to his laboratory at the Sloan-Kettering Institute in New York. During our first conversation Dr. Old suggested that I join Hiroshi Shiku, another postdoc, to study the mechanism of target cell killing by T cells. Dr. Old proposed to use a panel of antisera known to react with different surface antigens on thymocytes, to find if they could block cytotoxicity and in this way learn about possible involvement in this process of thus identified molecules. Dr. Shiku had just applied to the mouse system a new *in vitro* method, developed to evaluate cytotoxicity of lymphocytes by measuring the remaining radioactivity of adherent target cells labeled with tritiated proline (9). The specific antisera were provided by Dr. Boyse, among them anti Ly1 and anti-Ly2. The only information we had at that time about their reactivity with lymphocytes in different lymphoid organs was derived from *in vitro* absorption assays, which indicated that thymus had higher absorption capacity than lymph nodes and lymph nodes higher than spleen (8) correlating with the number of Thy1 positive cells in these tissues. My task was then to test the antisera against the effector cell population to determine their optimal titers using a complement dependent, trypan blue exclusion cytotoxicity assay.

Our repeated attempts to block cytotoxicity by pre-incubation of allo-reactive effector cells from immunized mice were unsuccessful[1] but because I noticed that the proportions of lymphocytes lysed by Thy1, Ly1, and Ly2 antisera in lymph nodes, spleen, and thymus were slightly different, we decided to test the effect of elimination of the cells sensitive to these antisera on the ability of surviving cells to kill target cells. The results showed that elimination of cells with Ly2 antisera (which lysed less lymphocytes than Ly1 antisera) inhibited cytotoxicity significantly stronger than elimination of lymphocytes with Ly1 antisera, suggesting that cytotoxic T cells can be distinguished from other T cells by their Ly1$^{low(-)}$Ly2$^{high(+)}$ surface phenotype. Some doubts, however, still remained because the methods used were not sufficiently precise to be sure that the relatively small differences we observed were real. Moreover, since we only tested cytotoxicity (10), the proof of functional heterogeneity of T cells was missing. In order to find whether Ly1$^-$2$^+$ and Ly1$^+$2$^-$ lymphocytes may have different functions, I approached John Hirst (who in the Herbert Oettgen laboratory studied the helper activity using

[1]Our failure to block cytotoxicity by Ly2 antisera was probably due to the idiosyncratic properties of our method since some years later E. Nakayama and colleagues achieved blocking by measuring chromium release from labeled non- adherent target cells. (Nakayama, E., Shiku, H., Stockert, E., Oettgen H. F. and Old L. J. Cytotoxic T cells: Lyt phenotype and blocking of killing activity by Lyt antisera. (1979) *Proc. Natl. Acad. Sci.* 76: 1977–81).

the Mishell-Dutton cell culture method) and proposed to test the effect of elimination of lymphocytes with Ly antisera on this regulatory function. Already, the first experiment produced clear-cut results: we observed practically no inhibition by treatment with Ly2 antisera and almost complete inhibition with Ly1 antisera, indicating that helper and killer T cells have different Ly phenotypes: $Ly1^+2^-$ and $Ly1^-2^+$, respectively. Importantly, I found that lymphocytes with different sensitivity to Thy1, Ly1, and Ly2 antisera are also present in the thymus of non-immunized normal mice, strongly suggesting that lymphocytes with different Ly phenotypes are generated independently of antigenic stimulation. These findings (11) represented the first unequivocal demonstration of functional heterogeneity of T cells and identified – as subsequent studies showed – their two major subsets. Moreover, identification of cells with three different Ly phenotypes ($Ly1^+2^+$, $Ly1^+2^-$, and $Ly1^-2^+$) in the thymus (11, 12) opened new possibilities to study T cell development and intrathymic mechanisms of selection. Years later monoclonal antibodies ousted the use of antisera. CD4, the newly discovered T cell specific molecule (13, 14) and functional counterpart of Ly2 (CD8) on $Ly2^-$ T cells turned out to better define T cell subsets than Ly1(CD5), which was later shown to be also expressed at a low level on $Ly2^+$ T cells and on some B cells (15). Consequently, subsets originally defined by Ly antisera as $Ly1^+2^-$ and $Ly1^-2^+$ became re-defined and re-named as $CD4^+8^-$ and $CD4^-8^+$.

Our results, which I publicly reported for the first time in summer 1974 at the workshop chaired by Martin Raff during the International Congress of Immunology in Brighton, UK, were obtained between December 1972 and August 1973. Although immediately recognized as very important (Dr. Boyse in his letter to WHO, dated August 7, 1973 wrote: "*Dr. Kisielow together with two other members of our group here has begun what may be a very important finding in the immunology of lymphocytes. Identification of different lines of T lymphocytes that may perform different function is a crucial step in our understanding of immunological responses and hence is of relevance to cancer no less than to several other fields of medicine*") the publication in Nature (11) was delayed by non-scientific reasons.

After my return to Poland, Harvey Cantor repeated, confirmed, and extended our results by providing firm evidence for antigen independent generation of $Ly1^+2^-$ and $Ly1^-2^+$ subsets (12). In addition, he showed that $Ly1^+2^-$ lymphocytes cooperate not only with B cells but also with $Ly1^-2^+$ lymphocytes in the generation of killer activity and obtained the first suggestive evidence that antigen recognition by $Ly1^+2^-$ and $Ly1^-2^+$ may be restricted by different classes of MHC antigens (16). This was around the time of discovering the MHC restriction phenomenon (17) and some time later it was firmly established that antigen recognition by $CD4^+8^-$ and by $CD4^-8^+$ T cells is restricted by class II and class I MHC molecules respectively, and that CD4 and CD8 molecules specifically interact with restricting MHC molecules on antigen-presenting cells (18). Puzzling, for a long time unanswered questions concerned the identity of the immediate precursor cells of $CD4^+8^-$ and $CD4^-8^+$ lineages and the mechanism of their intrathymic selection. Many researchers, including myself (19), tried to decipher the developmental potential of the obvious candidate cell, i.e., $CD4^+8^+$ ($Ly1^+2^+$) thymocyte. My desire to study this problem started my long time collaboration with Harald von Boehmer, who generously kept inviting me, and my students, to the Basel Institute for Immunology. There, we were fortunate to participate in the project aimed at studying T cell development and selection in TCR transgenic mice, which led to the identification of $CD4^+8^+$ thymocytes as a target of positive (20–22) and negative (23, 24) selection allowing us to gain insight into the mechanisms of these processes. Learning how T cell subsets, which we had identified 15 years earlier (11) are born in the thymus was one of the happiest experience in my scientific life, despite being aware that efforts to understand the immune system represent a continuous, never-ending asymptotic process where the answer to the given question is as good as the number of new questions it generates.

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Revisiting the first long-term culture of antigen-specific cytotoxic T cells

Kendall A. Smith*

The Division of Immunology, Department of Medicine, Weill Medical College, Cornell University, New York, NY, USA
**Correspondence: kasmith@med.cornell.edu*

Edited by:
Michael Loran Dustin, Oxford University, UK

Reviewed by:
Ellis L. Reinherz, Dana-Farber Cancer Institute, USA
Michael Loran Dustin, Oxford University, UK

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Prior to the publication of this article [link to Ref. (1)], immunological dogma held that lymphocyte proliferation was only initiated and sustained by antigenic (or mitogenic) stimulation. In support of this notion, several investigators had reported that repetitive stimulation of T cells with allogeneic lymphocytes in a mixed lymphocyte culture could result in the long-term culture of alloreactive T cells for several months (2–5). With the re-addition of irradiated allogeneic stimulator cells, usually every 2 weeks, a burst of proliferation would ensue, followed by a gradual tapering of the proliferative rate of the cell population until a subsequent re-stimulation. The cellular response was assumed to be entirely antigen-driven, such that as the irradiated allogeneic stimulator cells gradually died out, the responder cells naturally defaulted to a quiescent state, since there was no longer an antigenic drive.

However, even before these reports, a decade of publications, the first in 1965, indicated that the allogeneic or mitogenic stimulation of T cells "conditioned" the media, presumably by activating the cells to release soluble mitogenic "factor activities" for T cells (6–13). At the time, the molecules responsible for the putative activities remained totally obscure, in that the available analytical and preparative biochemical methods were fairly rudimentary.

In addition to T-cell mitogenic activities, Martin Cline and David Golde had reported that human lymphocytes stimulated with phytohemagglutinin (PHA)-conditioned media that would promote the formation of granulocyte and monocyte colonies in soft agar (14). Then, Doris Morgan, a hematopoietic biologist who had been searching for a factor activity that might sustain long-term leukemia cell proliferation, reported that PHA-stimulated human lymphocyte-conditioned media promoted not leukemia cell growth, but long-term T-cell proliferation (15). It was somewhat of a surprise that mitogenic activities from lymphocyte-conditioned media could select for and support the continuous culture of human T cells for as long as 13 weeks. However, as the cellular source was bone marrow, it was assumed by many that the T cells might be immature precursors, and not mature antigen-reactive T cells.

At the time, we were in the process of examining ways to promote the generation of cytotoxic T-lymphocytes (CTL) capable of lysing murine leukemia cells. Working under the hypothesis that allogeneic leukemia cells might promote an enhanced generation of CTL lytic not only for allogeneic but also syngeneic leukemias, we had already found that secondary and tertiary allogeneic mixed tumor-lymphocyte cultures markedly augmented the lytic efficiency of CTLs, especially against syngeneic leukemia cells (16). Even so, we had not tried to culture our CTLs beyond several days, assuming that they would progressively die out. However, given Morgan's report, we hoped that after an initial antigenic stimulation we might use lymphocyte-conditioned media to sustain long-term CTL proliferation with maintenance of lytic activity. Because concanavalin-A (Con-A) is a more potent mitogen for murine vs. human T cells, we produced Con-A T-cell supernatants, which one immunologist derisively abbreviated CATSUP, and seeded cell populations with CTL activity into 50% CATSUP. After several failed attempts by everyone in the lab, Steven Gillis, a graduate student and neophyte in cell culture, found that cells could be maintained in long-term culture provided they were seeded at low cell densities, $\sim 5 \times 10^3$ cells/mL, and never allowed to surpass $\sim 5 \times 10^5$ cells/mL (1). We speculated that perhaps low cell densities were necessary because the growth factor(s) in the CATSUP became limiting. Perhaps the high cell densities somehow consumed the activities.

The CTL lines (CTLL) retained their lytic activity upon repeated testing, and even increased their lytic efficiency up to 10-fold over several weeks of culture. Moreover, the CTLL expressed θ-antigens as expected for murine T cells, and tested negative for various histochemical stains specific for myeloid cells. Wright–Giemsa staining revealed lymphoblasts and highly vacuolated cytoplasm. Other tests at the time revealed electron-dense granules, which were subsequently found to be the cytolytic granules containing perforin and granzymes, the molecules responsible for T-cell cytolysis.

These findings were provocative for several reasons. First, the data were against the dogma that only antigens were responsible for T-cell proliferation. The data were also contrary to the notion that differentiated T-cell lytic activity and proliferation were mutually exclusive, as well as against the Hayflick hypothesis that normal cells, as opposed to malignant cells, were limited to ~50 cellular divisions before senescence ensued (17). Moreover, because the CTLL were capable of lysis of syngeneic leukemia cells, we speculated that it might be possible

to generate human CTLL via allogeneic mixed tumor-lymphocyte cultures, which then could be expanded *in vitro* with CATSUP and used as adoptive immunotherapy to actually treat human leukemia.

Accordingly, we proudly submitted our manuscript to *Nature*. We felt that this paper was worthy of such a prestigious journal as *Nature* because we had shown that it is possible to culture antigen-specific functional cytolytic T cells apparently indefinitely without antigen, which was totally against the dogma that antigen was solely responsible for lymphocyte proliferation. Also, we had succeeded in selecting and culturing not only antigen-specific CTL, but also tumor antigen-specific CTLL, a holy grail of tumor immunology. However, almost by return post we received our manuscript back un-reviewed and rejected. The terse form letter from the editor said that because *Nature* received so many excellent manuscripts they could not possibly review all of them. I personally was so incensed that I immediately fired off a letter to the editor, explaining why this particular manuscript should be reviewed "by someone with more than a cursory education in immunology." The good part of this story is that they reviewed and accepted our manuscript without changes, and it appeared on Bastille day in 1977.

This article eventually led to the generation of the first cytolytic T-cell clones, which will be the subject of another article in the series of "living immunological history" (18). However, we knew that we had difficult work ahead of us, in that our CATSUP, which was essential for our successful culture of CTLL, contained both a mitogenic lectin as well as any putative molecule(s) with soluble mitogenic activity. Which of the two was actually responsible for initiating and sustaining long-term T-cell growth had to be left to the future.

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Revisiting the discovery of the αβ TCR complex and its co-receptors

Ellis L. Reinherz*

Laboratory of Immunobiology, Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA, USA
**Correspondence: ellis_reinherz@dfci.harvard.edu*

Edited by:
Kendall A. Smith, Weill Medical College of Cornell University, USA

Reviewed by:
Salvatore Valitutti, INSERM, France
Nick Gascoigne, National University of Singapore, Singapore

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This is an opinion article based on the paper "Clonotypic structures involved in antigen-specific human T cell function. Relationship to the T3 molecular complex", by Meuer S. C., Fitzgerald K. A., Hussey R. E., Hodgdon J. C., Schlossman S. F., and Reinherz E. L. (1).

Life and science do not necessarily follow a straightforward path. So, it is not surprising, in retrospect, that my decision around Christmas of 1977 to terminate my clinical hematology fellowship in favor of a laboratory research associate position at Dana-Farber Cancer Institute working on lymphoid malignancies would result in progress in basic immunology. Remarkably, however, the outcome of the research yielded new insights into thymic development, mature T-cell heterogeneity, and the molecular basis for cognate recognition by T lymphocytes.

At the time, my decision was motivated by a clinical observation and desire to understand its basis. Namely, if a physician treated 100 children with acute lymphoblastic leukemia (ALL) using the same multi-agent chemotherapy, 80 of them would go into remission and 20 of them would die. I asked myself if that latter outcome was 100% of 20% vs. 20% of 100%. I thought that the answer might be the former but sought the opinion of a clinical mentor. He looked at me as if I had three heads and simply commenting that those who die have "poor protoplasm." This abject ignorance was so appalling and annoying to me at the same time that I quit on the spot. Since I had bluntly told him what I thought of his response, I likely would have been fired if I had not voluntarily chosen to move on down the road quite literally, as it were. While I had no explicit experimental plans, it occurred to me that antibodies raised against these tumor cells might be capable of distinguishing subpopulation heterogeneity, should it exist. After all, in 1977, people were not distinguishing red blood cells from different individuals by holding them up to the light. In the 1920s, a man named Landsteiner sorted out differences among RBCs that looked alike through development of red blood cell typing technology (2).

In any event, I moved to Dana-Farber Cancer Institute to work with Stuart Schlossman, who was Chief of the Division of Tumor Immunology, which included his own laboratory, that of Harvey Cantor, and several others. Stu enthusiastically greeted me at the time. He was appreciative of my query since he, himself, was trained as a hematologist and attempting to dissect normal lymphoid heterogeneity. His laboratory already was generating rabbit antisera against various types of hematopoietic cells. He told me that he was just starting the production of monoclonal antibodies (mAbs) using the Kohler and Milstein method (3) and that I might get involved. I retrieved human thymuses from children and neonates undergoing open heart surgery for congenital cardiac abnormalities, peripheral T-cells from normal volunteers isolated by sheep erythrocyte rosetting (a way of fractionating human T-cells), and a host of leukemic populations from my earlier patients. We used human cells to immunize mice in an effort to stimulate antibody production. The combination of the species differences as well as FACS-based screening of B-cell hybridoma supernatants *in lieu* of radioimunoassays employed by most other groups permitted us to quickly identify antibody targets, even when not expressed at high levels on cells or restricted to a subpopulation of those cells being interrogated. As a consequence, it was rather simple to derive mAbs against targets on subpopulations of mature and immature T lineage cells.

In 1979, we first identified the CD4 molecule, which we found to be expressed on two-thirds of peripheral mature T lymphocytes with helper activity (4–6) and then CD8 molecules expressed on the reciprocal subset of T-cells, which manifest most of the cytotoxic activity (7). In contrast, within the thymus itself, we originally described the major population of thymocytes co-expressing CD4 and CD8, which we termed double positive (DP) as precursors of mature thymocytes (8). In addition, we observed a small subset of $CD4^{-}CD8^{-}$ (double negative, DN) thymocytes. The vast majority of ALLs refractory to chemotherapy (those 20% above) was derived from the DN thymocytes (8). Susceptibility of this DN thymocyte population to activating mutations in NOTCH and aberrations of competitive niches created during early development are currently evolving as explanations of thymocyte susceptibility [(9) and references therein]. The fact is even without detailed molecular understanding of these immature ALLs and more mature T lineage malignancies like acute lymphoblastic lymphoma and Sezary syndrome, it was obvious that such tumors represented frozen states of normal T lineage development. The notion that thymocyte development progressed from DN to DP to

SP ($CD4^+CD8^-$ or $CD4^-CD8^+$) derived from our studies. Acceptance of this idea had to wait more than 4 years for the mouse immunologists to create the anti-murine CD4 mAb equivalent, L3T4 (10). The use of these mAbs revolutionized mature T-cell subset characterization in human beings offering CD4/CD8 clinical ratios, absolute CD4 counts, and the like. Comparative analysis done with Lorenzo Loretta and Max Cooper using other then currently accepted methods revealed that the new approach to define T lymphoid heterogeneity with mAbs was superlative to those existing technologies (11).

In 1980, we first observed that the anti-CD3 mAb could block antigen-specific human T-cell proliferation to both soluble antigens and alloantigens, as well as inhibit generation of cytotoxic T-cells (12). Of note, CD3 was expressed at latter stages of thymic development but maintained on all mature peripheral T-cells. Moreover, antigen recognition by human T lymphocytes was linked to surface expression of the CD3 molecular complex. When human T-cell clones were incubated with anti-CD3 mAb at 37°C, there was a rapid and selective loss of CD3 expression and concomitant antigen unresponsiveness (13). The latter was not a generalized cellular inhibition since IL-2 responsiveness remained intact. Removal of anti-CD3 from cell culture mAb was followed by restoration of T-cell surface CD3 expression and, in parallel, return of antigen responsiveness. These data set the stage for Ortho/Johnson & Johnson Pharmaceuticals to develop OKT3 as a human immunosuppressive therapeutic that was tested in treatment of allograft transplant rejection and became the very first FDA approved mAb in 1985 (14, 15).

Because research productivity went well, I was promoted to Assistant Professor in the Medicine Department at Harvard Medical School in 1980. Baruj Benacerraf had both taken over the helm of DFCI that year as its president and won the Nobel Prize in Physiology or Medicine in 1980 with Jean Dausset and George Snell for "their discoveries concerning genetically determined structures on the cell surface that regulate immunological reaction," i.e., MHC. As a newly minted faculty member, Baruj summoned me to his office so that I could describe to him the plans for my fledgling research operation. I told Dr. Benacerraf that all of my efforts were going to focus on defining T-cell antigen recognition, including identification of the T-cell receptor. He told me that this goal was ambitious, bold but probably ill advised. There were too many established laboratories working on this central immunological problem. "What makes you think it's likely that you will succeed over them?" he queried. I responded: "I'm looking for the receptor on the surface of a T cell, not in culture supernatants as the others are doing." He shrugged, wished me luck, and so the next phase began. In the several years that followed when success was achieved, it should be noted that Baruj was laudatory and glad I followed my scientific conviction.

In 1982, by exploiting T-cell cloning techniques, first described by Smith and colleagues (16), alloreactive CTL could be derived from both human CD4 and CD8 subsets. Strikingly, CD4 T-cells recognized MHC class II products, whereas CD8 T-cells recognized MHC class I products. These cells could be blocked by appropriate anti-MHC I/II or anti-CD4 or anti-CD8 antibodies (17–22). Given that anti-CD3, anti-CD4, and anti-CD8 mAbs all blocked CTL activity, we wondered whether the surface molecules identified by these mAbs detected recognition elements or, alternatively, components of the lytic machinery. Lectin approximation studies excluded the lytic machinery option since even in the presence of the blocking antibodies, lectin restored CTL function, although pointedly with loss of target specificity.

The fact that biochemical analysis [see, for example, in Ref. (23, 24)] failed to identify differences in peptide maps or electrophoretic mobility of these molecules on human T-cell clones of differing specificities argued that they were invariant structures incapable of conferring antigen and MHC specificities *per se*. Because each cloned T lymphocyte recognizes antigen in a precise fashion, one could not account for its unique specificity on the basis of monomorphic portions of CD3, CD4, or CD8 (1). I reasoned that there had to exist discriminative surface structures on individual clones, which we refer to as clonotypes or idiotypes. mAbs to such idiotypic structures (T idiotypic = Ti) were produced next by immunizing mice with CTL clones, screening the resulting antibodies on the immunizing CTL and then selecting those which lacked reactivity with additional clones of different antigen specificities from the same donor (1, 25, 26). Such antibodies were unique in that they inhibited cell-mediated killing and antigen-specific proliferation of the individual immunizing clone without affecting the function of other autologous clones. Moreover, like anti-CD3 mAbs, the anti-clonotypic antibodies enhanced IL-2 responsiveness and induced modulation of the Ti structure with CD3. Data showed that the Ti clonotype was closely associated with CD3 in the membrane of human T-cells. Immunoprecipitation and competitive binding analysis revealed that the anti-clonotypes defined a disulfide-linked heterodimer with α and β subunits of approximately 49 and 43 kD, respectively. The heterodimeric clonotype was not physically associated with CD4 or CD8 but was in non-covalent association with the invariant CD3 molecules as a complex first evidenced by our analyses (1, 23, 25, 26).

From the above data collectively, I proposed with my colleagues a working model of T-cell cognate recognition (27) in which the antigen binding structure comprised a clonally unique αβ heterodimeric Ti moiety in complex with CD3. The associative recognition element is either CD4 or CD8 depending on the subset derivation of the individual T lymphocyte. In this model, CD4 and CD8 accessory ("co-receptor") glycoproteins bind to constant regions of class II or class I MHC, respectively, which are separate from the CD3-linked clonotype. The TCR complex, on the other hand, was defined as a CD3-associated Ti αβ heterodimer working in concert with CD4 and CD8 to mediate MHC-restricted antigen recognition. This view implied that there was a bidentite interaction of the TCR complex and co-receptor with the same peptide/MHC. This proposal has been codified in structural studies over the last 30 years [for review, see Ref. (28)]. Confidence that Ti was the αβ TCR heterodimer encoding both peptide and MHC specificities came from (1) the unique ability of anti-clonotypic mAbs coupled to Sepharose beads to trigger T-cell clones, replacing requirements for cognate antigen plus MHC (29); (2) biochemical evidence for peptide variability within α and β subunits of Ti (30, 31), implying existence of constant and variable regions as found in

Ig heavy and light chains; (3) αβ purification and amino acid sequencing showing Ig homologies for each subunit (32, 33); (4) the putative TCR triggering resulted in T-cell proliferation via an IL-2-dependent mechanism not observed by crosslinking other T-cell structures (34); and (5) direct evidence for the existence of nominal antigen binding sites on Ti αβ heterodimers of MHC-restricted T-cell clones specific for fluorescein-5-isothiocyanate (35).

The work carried out over a period of these several years was extraordinarily exciting, converting concepts into explicit molecular identities, beginning to explain the complexities of T-cell recognition, providing reagents for clinical efforts and fodder for considerable future structural and molecular studies. These TCR efforts required a spirited collection of colleagues including Stefan Meuer who developed T-cell clones and performed many functional studies with Rebecca Hussey who made the various mAbs used in the majority of these studies, and Oreste Acuto who led the biochemical charge on the TCR complex and TCR αβ heterodimer purification and amino acid sequencing with Marina Fabbi. Bob Siliciano then showed that the TCR αβ heterodimer actually bound ligand.

Our efforts on TCR biology and its identification were complemented very soon by studies in the mouse by Pippa Marrack and John Kappler using T-cell hybridomas. More explicitly, we published in JEM in February 1983 on the first anti-clonotypic mAb (1), while they published in the same journal in April 1983 (36). Comparisons of TCR αβ heterodimer clonotypes were published by us in Nature in June 1983 and in PNAS in July 1983 (23, 25) whereas their comparison appeared in Cell in October 1983 (37). We published on the ability of ant-clonotypic mAbs to replace the requirement of peptide and MHC in T-cell activation in September 1983, whereas our competitors showed that anti-clonotypic antibody binding to T-cell hybridomas predicted antigen and MHC specificity in November 1983 (38). Furthermore, their biochemical data matched well with that of the human being and an independently identified disulfide-linked T-cell tumor-specific antigen identified with a mAb produced by Allison et al. (39). The human being was a particularly informative and tractable species choice since we had created reagents that defined the TCR αβ heterodimer, CD3 components, and CD4 and CD8 co-receptors. The majority of reagents defining these receptors was lacking in the mouse at the time. Biochemical detail by Terhorst and Klausner further refined the nature of the CD3 components of the TCR complex (40, 41). The objective impact of the three Reinherz, Marrack, and Allison group efforts from the 1980 to 2000 time period is evident from ISI citations (20,000 vs. 8,000 vs. 900, respectively). In turn, molecular cloning of the TCR subunits using a subtractive hybridization method began (42–45). These studies identified TCRβ as shown by our subsequent N-terminal amino acid sequencing analysis (32). More than 30 years later, however, we are still in the process of detailing structure and function of the TCR complex and its co-receptors. The trove of information herein will lead to important therapeutic inventions for treatment of autoimmune and immunodeficiency diseases to be fully realized in the coming years.

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GENERAL COMMENTARY
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Commentary: Production and characterization of monoclonal antibodies to human interleukin 2: strategy and tactics

*Kendall A. Smith**

Division of Immunology, Department of Medicine, Weill Medical College, Cornell University, New York, NY, USA

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A commentary on

Production and characterization of monoclonal antibodies to human interleukin 2: strategy and tactics
*by Smith KA, Favata MF, Oroszlan S. J Immunol (1983) **131**:1808–15.*

Although we had successfully created antigen-specific cytolytic T lymphocyte lines (CTLLs) using conditioned media as a source of growth-promoting factors, we had no effective method to determine the relative activities of different batches of conditioned media. Therefore, it was crucial to create a quantitative assay for the activity we termed "T cell growth factor" (TCGF). Fortunately, Torgny Fredrickson and I had already created a bioassay for the red blood cell growth factor, erythropoietin (EPO), using murine fetal liver cells that are enriched for EPO-responsive precursors (1, 2). Thus, patterned on the EPO bioassay, it was straightforward to construct a similar assay for TCGF using as target cells our long-term CTLL. The critical elements of the assay were (1) a low density of CTLL target cells and (2) serial twofold dilutions of conditioned media samples, thereby establishing a dose–response curve that allowed comparison of separate conditioned media (3). Of note was the observation that the curve was symmetrically sigmoid when the linear responses of tritiated thymidine incorporation were plotted vs. the logarithm of the conditioned media dilutions. We arbitrarily assigned 1.0 U/mL that yielded 50% of maximal growth promotion at a dilution of 1:10. This assay represented the first ever quantitative bioassay for a lymphokine.

Thus, armed with a rapid, quantitative bioassay, we next sought to generate T cell clones derived from our antigen-specific CTLL so that we could assess the potential problem of target cell heterogeneity. We tried two established cloning methods: (1) dilute cell suspensions seeded into soft agar containing TCGF-conditioned media and (2) limiting dilution (0.03–0.01 cells/well) in microtiter plates containing TCGF-conditioned media. The limiting dilution technique in suspension culture worked very well, yielding 67–100% plating efficiency. This was the first description of monoclonal antigen-specific cytolytic T cells (4). Accordingly, T cell clones permitted an unambiguous interpretation that TCGF was acting directly on cloned T cells and not indirectly through an intermediate cell type, e.g., an APC. We submitted our manuscript to *Nature*, which again rejected it without review [see Ref. (5)], so that we immediately reformatted it and sent it to the *J. Exp. Med.*, which accepted it without changes, so much for non-scientist journalists (Nature) vs. peer scientists (JEM) making informed editorial decisions (6). Other investigators rapidly adopted these cloning methods, in that they not only allowed for separation of the cell clones, but also could be used to grow large numbers of progeny, which could be used for both biological and molecular characterizations. The ability to create monoclonal functional T cells was as transformative for T cells as monoclonal antibodies were for B cells.

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Rene De Waal Malefyt, Merck Research Laboratories, USA

Reviewed by:
Gerard Zurawski, Baylor Institute for Immunology Research, USA

***Correspondence:**
Kendall A. Smith
kasmith@med.cornell.edu

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The foregoing results of these studies on the various mitogenic activities in conditioned media pointed to the overwhelming need to identify the molecules responsible for the bioactivities. In addition, the molecular mechanisms whereby the mitogenic activities interacted with their target cells loomed as a huge overriding question. Thus, armed with the quantitative TCGF bioassay, analytical biochemical experimental approaches yielded results consistent with a single, small protein (~15.5 kDa; pI = 8.2) as solely promoting the biological response (7). This was an important finding, in that it meant that further purification of the molecule responsible for the activity would be straightforward, whereas if several molecules cooperated to produce the activity, purification of each component would be difficult.

These biochemical approaches permitted the separation and purification of enough biosynthetically radiolabeled TCGF to permit classic hormone binding assays, which revealed that radiolabeled TCGF-binding sites expressed all of the characteristics of true hormone receptors, i.e., the binding was restricted to TCGF-responsive cells, there was a lack of competition by other growth factors and hormones, the binding was of very high affinity, and there was a close correlation between the TCGF concentrations that bound to cells and those that mediated the proliferative response (8). These data all supported the conclusion that the binding site detected was on the receptor through which the biological effects of TCGF are initiated. This report was the first to demonstrate and characterize a cytokine receptor, and consequentially became the prototype for the identification and characterization of all subsequent cytokine receptors involved in regulating immune and inflammatory responses. Moreover, the radiolabeled TCGF binding assay was absolutely instrumental in the identification of the first monoclonal antibody reactive with a cytokine receptor molecule (9).

These experimental approaches allowed us to calculate the Specific Activity of IL-2 for the first time, so that 1.0 U/mL = 150 ng/mL, which indicated that we would need to start with several liters of conditioned media to concentrate and purify adequate IL-2 protein to immunize mice with microgram amounts of TCGF protein and ultimately to develop the first monoclonal antibodies reactive with a lymphokine molecule (10). Prior to the development of quantitative bioassays and radioreceptor assays, investigators had tested only one dilution of a sample, e.g., a 1:2 or a 1:4, so that they could not quantify the amount of activity and relate it to a measured protein concentration. Thus, attempts were made to purify cytokine molecules with only 10–100 mL of starting conditioned media. In this article, we detailed the critical experimental approaches and advances that led to our success in generating monoclonal antibodies reactive with human TCGF, so that others might follow. Through rigorous biochemical analytic methods, it was possible to prove that the monoclonal antibodies were useful to immunoaffinity purify IL-2 molecules to homogeneity in milligram quantities from multiple liters of conditioned media that then could be used for unambiguous molecular and biological characteristics of the first interleukin molecule to be identified.

Thus, with these new and novel cellular and molecular reagents, we could proceed to experiments that had never been done before.

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Commentary: "The role of T3 surface molecules in the activation of human cells: a two-stimulus requirement for IL-2 production reflects events occurring at a pretranslational level"

Arthur Weiss[1]* and John D. Stobo[2]

[1] *Department of Medicine, Rosalind Russell-Ephraim P. Engleman Rheumatology Research Center, Howard Hughes Medical Institute, University of California, San Francisco, CA, USA*
[2] *Office of the President, University of California, Oakland, CA, USA*
**Correspondence: aweiss@medicine.ucsf.edu*

Edited by:
Kendall Arthur Smith, Weill Medical College of Cornell University, USA

Reviewed by:
Ellis L. Reinherz, Dana-Farber Cancer Institute, USA
Barbara Fazekas De St Groth, Centenary Institute of Cancer Medicine and Cell Biology, Australia

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A commentary on

The role of T3 surface molecules in the activation of human cells: a two-stimulus requirement for IL-2 production reflects events occurring at a pretranslational level
by Weiss A, Wiskocil R, Stobo J. J Immunol (1984) ***133****:123–128.*

In 1982, identifying the T cells antigen receptor was still the elusive "Holy Grail" of immunology. However, the ability to use monoclonal antibodies (mAbs) to identify and characterize molecules on lymphocytes, coupled with the then recent ability to grow long-term antigen-specific T cell clones, provided a strategy to identify the TCR by isolating clone-specific mAbs. We decided to take on this ambitious, but exciting project.

We set out to grow allo-reactive human T cell clones with different specificities in order to use one clone as an immunogen and other clones with different specificities as controls. To grow human T cell clones, a source of growth factors to maintain long-term T cell clones was needed and the recently identified interleukin-2 (IL-2) was the best candidate. However, *human* T cells required *human* IL-2 for their propagation and it was going to be cumbersome to stimulate large numbers of human peripheral blood T cells for a source of the growth factor. The IL-2 gene had only recently been cloned and recombinant IL-2 was not available. However, work from Gillis and Watson described a human acute lymphoblastic leukemic T cell line called Jurkat that could be stimulated with phytohemagglutinin (PHA), a mitogenic plant lectin reactive with carbohydrates, to produce IL-2 (1). Stimulating large numbers of Jurkat cells to produce IL-2 for T cell cloning purposes offered a simple solution to our dilemma. We were able to obtain the Jurkat line from Kendall Smith and it seemed our problem was solved. PHA could stimulate the line to produce moderate amounts of bioactive IL-2 and the amount that it produced could be boosted by the addition of the tumor promoter, phorbol myristate acetate (PMA).

Just as we were getting started on the project, we were dealt a crushing blow by a paper from Meuer et al., who used T cell clone specific mAbs to convincingly identify the TCR (2). They found a clone specific αβ heterodimer on a human T cell clone. The identification of the αβ heterodimer as the TCR was consistent with a tumor-specific structure that had previously been identified by Jim Allison's group, who had speculated that the tumor-specific heterodimer might potentially represent the TCR (3). Importantly, Meuer et al. also suggested that the clone-specific heterodimer that they identified was associated with the T3 (later named CD3) complex (2), whose expression was previously linked to antigen-specific recognition (4). It had been known for a few years that mAbs against T3, like PHA, were mitogenic and could substitute for antigen in inducing T cell activation (5, 6). As we regrouped, it occurred to us that since Jurkat cells could be activated by PHA, Jurkat might express T3 and even an antigen receptor. Indeed, we found that Jurkat expressed T3 antigens. In parallel studies, we went on to make clone specific mAbs to the Jurkat αβ heterodimer, including the IgM C305 which is a Vβ8 specific mAb commonly used in Jurkat studies today (7).

We considered the possibility that Jurkat might be stimulated via its TCR-T3 complex, but stimulation of the cell with only anti-T3 mAb resulted in no detectable secreted IL-2, as assessed by bioassay (note IL-2 was detected by bioassay using the IL-2 dependent clone CTLL-20). However, we found that the addition of PMA, which had boosted IL-2 production induced by PHA, converted a negative result into a very robust positive one (8). The IL-2 response was highly specific for mAbs to T3 combined with PMA. Moreover, we found this result to be quite interesting: two stimuli, one putatively involving a component of the TCR complex, were required for the Jurkat line to produce IL-2.

We wondered how these two stimuli operated in concert to induce IL-2 production. Fortunately, we had an experienced molecular biologist in the lab, Bob Wiskocil, with whom to collaborate to address this question. Using Northern blot and dot blot analysis, Bob showed that

the combination of PHA and PMA could induce the accumulation of abundant IL-2 transcripts in Jurkat, whereas PHA alone induced only low levels of IL-2 RNA (8). More interestingly, while neither OKT3 (an anti-T3 mAb) nor PMA induced detectable IL-2 RNA, the combination induced very robust accumulation of IL-2 transcripts. These results suggested that the two stimuli operated in concert to regulate the accumulation of IL-2 RNA.

Our results were consistent with work in the field involving complex mixtures of cells that suggested a two-signal requirement for IL-2 secretion or for T cell proliferation, but our studies simplified the study of this phenomenon by utilizing simple stimuli and a single cell type. Importantly, it provided a very simple experimental cell line model to study T cell activation of IL-2 production independently of T cell proliferation. Indeed, the Jurkat model, while having limitations, has proven extremely valuable as an experimental tool over the ensuing years with nearly 16,000 PubMed references for "Jurkat." Numerous other studies have used Jurkat mutants to study signaling and other phenomena (9). Many of the proximal molecules involved in TCR signaling have been either discovered or validated in the Jurkat system. Moreover, our results suggested complex regulation for the IL-2 gene at the transcriptional level requiring multiple signal inputs, a notion that has since been well validated and expanded upon by the variety of signal inputs that have since been shown to regulate the IL-2 promoter and its 3′ untranslated region (10–12).

The observation that two stimuli could induce Jurkat to produce IL-2 validated the concept that stimulation of the TCR alone was insufficient to activate T cells. Dating back to 1970, Bretscher and Cohn had proposed that stimulation of the antigen receptor was insufficient to activate a naive T cell (13). Much of the early work on the two-signal model had confounded the field by the complex mixtures of cells used and the complexity of the antigens, second signals, and varied assays used to assess T cell activation. Our work provided Jurkat as a simplified T cell model, IL-2 RNA accumulation or secretion of this cytokine as a simple output for assessing activation and a simple stimulus for triggering the TCR. This simple model system supported the two-signal hypothesis and elaborated some of the integration of the two signals at the level of IL-2 gene regulation. The second signal was not well addressed by our paper in the *Journal of Immunology*. Instead of a physiologic stimulus, we used a small organic molecule, PMA, to provide the second signal. PMA had recently been shown to activate a protein kinase, protein kinase C (PKC) (14), which was later shown to consist of a family of kinases (15). It was also later shown that PMA also activates the Ras pathway by virtue of it ability to activate the guanine nucleotide exchanger RasGRP (16). Abundant work from many labs has well documented the critical roles for the activation of PKC and Ras in the pathways leading to IL-2 gene transcription. A short time after our *Journal of Immunology* paper discussed here, studies from our lab and from others identified mAbs to Tp44 (later named re-CD28) on Jurkat and on normal human T cells as being capable of delivering the critical second or "costimulatory" signal for the production of IL-2 (17, 18).

The reductionist approach toward studying T cell activation using the Jurkat line invigorated the lab and led to an exciting time of discovery that included not only studies of the two signal model of T cell activation, but also included: (1) the discoveries that stimulation of T3 or the TCR led to an intracellular calcium increase (19, 20); (2) the demonstration that the calcium increase in the cytoplasm was the result of activation of the inositol phospholipid pathway (21); and, (3) the discovery that the expression of the T3 complex required co-expression of the TCR αβ heterodimer (7, 22). During these exciting times, we were joined by several colleagues in the Stobo lab but most noteworthy were the contributions of John Imboden and Bob Wiskocil. We would also like to thank Kendall Smith for generously providing the Jurkat cell line that led to an incredible time of discovery and collegiality in the Stobo lab.

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Commentary: The interleukin-2 T cell System: a new cell growth model

*Kendall Arthur Smith**

Division of Immunology, Department of Medicine, Weill Medical College, Cornell University, New York, NY, USA

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A commentary on

The interleukin-2 T cell system: a new cell growth model
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Having created unique and novel cellular and molecular reagents, we could approach questions that had perplexed investigators interested in cell growth for over 50 years, i.e., the basis for variable cell cycle transit times of individual cells among genetically homogeneous cell populations (1). Studies of all cell populations, both prokaryote and eukaryote, revealed that within a cell population, the cell cycle times of individual cells follow a normal distribution when examined as a function of the division rate (the rate-normal distribution). Thus, some cells proliferate slowly while others proliferate faster, with most cells distributed about the mean on a log-linear plot. Prior to the discovery of the IL-2 molecule (2) and IL-2 receptors (IL-2R) (3), T cell populations were known to follow this rate-normal distribution, common to all living cells. Mathematical analysis of proliferating cell populations indicated that individual cell variability in cell cycle transit times was stochastic, depending upon a "hidden variable." However, once the "hidden" molecular variables of T cell cycle proliferation had been identified (the IL-2 concentration, IL-2R density, affinity of the IL-2/IL-2R interaction, and the duration of the IL-2/IL-2R interaction), experiments could be preformed for the first time revealing that as long as these crucial characteristics of T cell cycle progression were known, then the variability of cell cycle transit times were entirely predictable and deterministic, not probabilistic or left to chance.

These experiments and approaches were possible because of painstaking attention to the creation of critical cell clones and homogeneous purified IL-2 molecules, as well as the development of the radiolabeled IL-2 binding assay, and monoclonal antibodies reactive with both IL-2 and its receptor. Moreover, the employment of the flow cytometer allowed us to proceed beyond studies of cell populations to quantify the intermolecular interactions of individual cells for the first time. Doreen Cantrell, a postdoctoral fellow skilled in flow cytometry, was critical to our experimental approach. Because the growth characteristics of all known cell populations are identical to those of T cell populations, it followed that individual cells of all other cell populations would demonstrate the same type of molecular determinants. Thus, the title of this article: "The interleukin-2 T cell system: A new cell growth model," a new universal paradigm in cell and molecular biology (1).

The significance of these findings was obvious. As cells of all tissues, especially of metazoans, have identical growth characteristics of T cells, it followed that the cells of all metazoans are regulated in the same molecular fashion, i.e., cells are directed to undergo all-or-none (quantal) cell fate decisions by critical molecular concentrations. Furthermore, as all malignant cells arise from a single cell, so that all malignancies are clonal in origin, a gain of function mutation of one of the genes encoding the molecular determinants of cell cycle progression could obviate the strict cytokine/receptor/signaling pathways normally controlling the decision to divide, thereby resulting in a neoplastic cell growth (4). In other words, a critical "driver mutation" can put the cell on "autopilot." From the viewpoint of the immune system, Burnet's "Clonal Selection Theory," derives

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Edited by:
Nick Gascoigne, National University of Singapore, Singapore

Reviewed by:
Omer Dushek, University of Oxford, UK

***Correspondence:**
Kendall Arthur Smith
kasmith@med.cornell.edu

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from the observation that antigen-reactive clones of immune cells are selected by antigen, but subsequent to selection, each clone undergoes a proliferative clonal expansion (5). It is this clonal proliferative expansion that is determined by the molecular parameters discovered in this series of experiments focused on IL-2 and T cells. Accordingly, the regulatory cells and other cytokine molecules that influence the concentrations of the IL-2 molecules, their receptors, and the molecules of their signaling pathways ultimately determine the tempo, magnitude, and duration of immune responses. That is, internally derived hormones and their receptors regulate the immune system, just as every other bodily system is regulated, and immunity is not regulated solely by environmental antigens from without, a previous central dogma of immunology.

For immunologists, it is especially noteworthy that the lymphocyte antigen receptors function in an identical fashion to convert TCR signals to a digital control of cytokine gene expression (6–10).

These findings and considerations, led me to propose "The Quantal Theory of Immunity" (11–14). Thus, the immune system is regulated at the systemic (population) level by the number of clones responding and the extent of the proliferative expansion of each clone as originally stated by Burnet. However, at the level of individual cells, participation in an immune response is regulated in a quantal (all-or-none) fashion, and this quantal decision is determined by the absolute number of intermolecular interactions, which the cells somehow "count." Once the crucial number has been surpassed, the cell responds in a quantal fashion. It goes without mentioning that all of the molecules and cells that participate in an immune response obey identical principles.

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Cloning CTL-specific genes (and now for something completely differential)

R. Chris Bleackley*

Department of Biochemistry, University of Alberta, Edmonton, AB, Canada
**Correspondence: chris.bleackley@ualberta.ca*

Edited by:
Nick Gascoigne, National University of Singapore, Singapore

Reviewed by:
Stephen Morrison Hedrick, University of California San Diego, USA
Anton Van Der Merwe, University of Oxford, UK

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The work described here began as a project to use differential hybridization to identify genes that were induced after T cell activation. In reality, it became something completely different when the approach identified genes that encoded proteins involved in the killing mechanism used by T lymphocytes (1, 2).

The late 1970s and early 1980s were exciting times of discovery in immunology. Lots of fascinating biological observations had already been made but there was little in the way of molecular understanding. During this period, the newly emerging tools of recombinant DNA technology and molecular biology were becoming more readily accessible and being applied to study immunological questions. This led to many exciting discoveries such as antibody and T cell receptor gene rearrangements (3–5).

I had some experience looking at antibody genes and the biochemical and genetic characterization of interleukin 2. Using these new approaches, I decided to investigate cytotoxic T cell (CTL) activation at the level of gene activation. The guiding hypothesis was that the phenotype of a cell was governed by the proteins expressed. We posited that each cell would express a set of function related proteins and their corresponding mRNAs. Consequently, we set about to identify mRNAs that were expressed specifically in activated CTL. At worst, we believed that this approach would uncover a set of interesting genes to study CTL activation at the transcriptional level. The real hope was that we would identify proteins involved in the killing mechanism. Interestingly, when this was reviewed for funding it was described as a fishing expedition. These days that would be the kiss of death for a proposal. However, these were more reasonable times and it was funded.

At the time, very little was known about how CTL induced death in target cells that they recognized. The effector cells were known to contain cytoplasmic granules that polarized toward the target upon conjugate formation. It was believed that they contained cytolytic proteins that induced membrane damage. One of the key molecules responsible for the ring-like lesion observed on target cells was ultimately shown to be cytolysin/perforin (6, 7).

We took advantage of the discovery of methods for the long term culture of CTL (8) and had generated a CTL line MTL2.8.2 (9). Using mRNA from this killer cell, a library of 4000 cDNA clones were generated. Individual colonies were picked and isolated in microtiter plates. Copies of these were replicated in triplicate onto nitrocellulose and grown. After lysis, the blots were hybridized with 32-P cDNA probe generated from MTL poly A^+ RNA. Positive colonies were identified and then the three copies of the library on the membrane were stripped and re-probed with radioactive cDNA prepared from a helper T cell line CH1. After autoradiography, the blots were again stripped and hybridized with probe from unactivated thymocytes.

The various autoradiograms were compared. Only colonies, which were strongly positive in two copies with the MTL probe and negative with CH1 and thymocyte cDNA, were selected. That gave us 121, which were rescreened yielding 36, which were clearly CTL specific. Two were chosen for further analysis; B10 was the most abundant and cross hybridized with eight other inserts and C11 because it was related to B10 but different. Tissue specificity cytodots clearly established that these two were CTL specific. The one exception was a signal in a suppressor T cell line derived from fetal thymus (10). This was not pursued further as the suppressor field was murky at best at this time. However, now it seems likely that this was a regulatory T cell.

The key observation was the correlation of mRNA expression of both B10 and C11 with cytotoxicity in both allogeneic and mitogen stimulated spleen cells. The level of killing measured in a chromium release assay peaked roughly 24 h after the maximum levels of both B10 and C11. In addition, as cytotoxicity declined, so did expression of the mRNAs (1). This was exactly the behavior expected for function related transcripts when it became very exciting.

A full length clone for C11 was isolated and sequenced. An open reading frame was identified, which predicted that the protein encoded was a serine proteinase most similar to the rat mast cell proteinases. Most important, the predicted protein contained the catalytic triad responsible for the enzymatic activity of this family of enzymes. A full length clone corresponding to B10 could not be found but sequence homology and sequence alignment predicted that it also encoded a proteinase.

At this time, I met Irv Weissman at a conference and discovered that his group had also cloned a serine proteinase gene from activated CTL. Sequences were compared and much to our delight that they were related but different. It was decided that the two manuscripts would

be submitted together to Science. Rather surprisingly, Irv's paper was accepted but ours was rejected. Only through Irv's direct intervention with the editor was this decision reversed and the two papers appeared side-by-side (2, 11).

They named their enzyme Hanukkah Factor while ours were christened cytotoxic cell proteinase 1 and 2 (CCP). Following the outstanding biochemical purification of a family of serine proteinases from CTL granule by Jurg Tschopp's group, they became more commonly called granzymes (12). Granzyme B (CCP1) was also named CTLA1 by Pierre Goldstein's group (13). The majority of these genes are clustered on mouse chromosome 14 close to the alpha-chain of the TCR (14).

The discovery of these enzymes created quite a stir of excitement in the field. Hudig and Redelman had suggested, for many years, a key role for proteinases in the killing mechanism. Pasternack et al. had recently demonstrated the importance of a trypsin-like enzyme that was associated with perforin containing granules and secreted upon target cell interaction (15). In addition, the groups of Podack and Henkart had purified the proteins responsible for the ring-like lesions seen in membranes treated with cytolytic granules. They named the molecules perforin (6) and cytolysin (7), but later it became apparent that they were the same protein. The general consensus was that the killing mechanism involved a proteinase cascade analogous to that seen in the complement system. The ultimate target of this cascade model was perforin, which upon activation would create the membrane damage observed.

We now know that perforin mediated target cell lysis is not the whole story. Rather cells under attack by CTL die via a mechanism involving DNA fragmentation. Perforin alone cannot mediate this form of death. It is now known that perforin facilitates the uptake and release of granzymes into the cytoplasm of the targets. The granzymes then cleave specific substrates that bring about the ultimate demise of the cell. Most notable is the cleavage of caspases by granzyme B that initiates apoptosis and brings about DNA fragmentation, but it took another 10 years to discover this (16, 17).

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A short history of the B-cell-associated surface molecule CD40

Edward A. Clark*

Department of Immunology, University of Washington, Seattle, WA, USA

Edited by:
Kendall A. Smith, Weill Medical College of Cornell University, USA

Reviewed by:
Bruce David Mazer, Montreal Children's Hospital, Canada
Kendall A. Smith, Weill Medical College of Cornell University, USA
Michael R. Gold, The University of British Columbia, Canada

****Correspondence:***
Edward A. Clark, 750 Republican Street, Room E343, Seattle, WA, USA
e-mail: eaclark@uw.edu

This perspective traces developments using monoclonal antibody technology that led to the discovery of CD40, a receptor that on B cells mediates "T cell help" and on dendritic cells helps to program CD8 T cell responses. I discuss some things that we got right during the path of discovery and some things we missed. Immunotherapies that block or stimulate the CD40 pathway hold great promise for treatment of autoimmune diseases and cancers.

Keywords: B cell, CD40, monoclonal antibody, activation, therapy

During the late 1970s, a number of immunology labs started using the new hybridoma technology to establish monoclonal antibodies (mAbs) and define receptors expressed at different stages of hematopoietic cell differentiation. A central premise of this field was based on the seminal studies of Ted Boyse, Lloyd Old, and their colleagues (1, 2). They used antisera to define what they termed "differentiation antigens" (Ags), which unlike histocompatibility (H) antigens, were a "source of antigenic variation within the species that between different cell types in a single individual" and thus were "recognizable only with antiserum prepared in a foreign species" (1). The groundwork was laid for trying to make mouse mAbs to differentiation Ags that could be used to define stages of lymphocyte differentiation and distinct functional cell subsets, an approach I used to make an mAb to what is now called CD40 (3).

As a postdoc in London with Av Mitchison, I had made mAbs, including some to the mouse T-cell marker, Thy-1. Upon arriving in Seattle, I decided to make mAbs to human B-cell differentiation Ags. To identify markers conserved in evolution, we immunized mice with a baboon B-cell line and screened for mAbs that reacted with human B-cell lines but not other lymphoid or non-lymphoid lines. One mAb that reacted just with B lymphoblasts but not T-cell blasts called BB1 (4), Peter Linsley later used BB1 to define CD80 (B7/BB1) as a ligand for CD28 (5).

We also immunized mice with human B cells and selected mAbs that bound to human B cells and not T cells. In 1982, we submitted a set of our mAbs to an international workshop established to classify mAbs binding to human leukocyte differentiation Ags. A major impetus for this first workshop came from scientists who had organized workshops to define and standardize HLA antigens and HLA typing. As had been the case for HLA nomenclature, a neutral nomenclature was needed for human differentiation antigens, particular since markers like CD4 were called different names-T4, OKT4, Leu3, etc., depending on the company that was hawking the mAb for sale. The HLA serologists proposed a serological approach to define so-called "clusters of differentiation" (CD) to identify and name human differentiation markers. I had started out working in a HLA serology lab with Paul Terasaki at UCLA. But I was influenced by a group of scientists with strong biochemical backgrounds, particularly by Jeff Ledbetter and also later by Tucker LeBien and Bob Knowles, who felt that it was very important to use a combination of biochemistry and serology to define the new CD molecules. So, we decided to use the molecular weight of any new molecule when naming it. One B-cell-restricted 35-kDa molecule we called Bp35 (later designated CD20), while another 76-kDa molecule expressed on activated lymphocytes we called p76 (later designated CD54/ICAM1). Using this approach, by late 1982, we were able to conclude that "there are now over 12 different human B-cell-associated antigens distinct from immunoglobulin" (6). What could all their functions be?

At the time of our discovery of CD40 (3), immunologists were just beginning to understand how B cells are activated to divide and differentiate. One widely held model was that, after an initial activation signal, e.g., by a low dose of Ag or other means of B-cell receptor (BCR) crosslinking, B cells were activated and then expressed receptors for growth and differentiation factors such as "B-cell growth factor" (BCGF) and "B-cell differentiation factor" (BCDF) derived from T cells, the precursors of IL-4, IL-6, and IL-21, so that in the presence of these factors, B cells would divide and differentiate (7, 8). A receptor for the "T-cell growth factor," IL-2, had been described (9), but not a BCGF receptor B-cell equivalent.

Boyse and his colleagues (10) and Subbarao and Mosier (11) had used mAbs to define a B-cell-restricted surface molecule in mice, Lyb-2 (later designated CD72), which when ligated with an mAb-induced B cells to proliferate but blocked their differentiation. Following this lead, we decided to test if any of our mAbs could activate human B cells. Geraldine Shu, a research

scientist in the lab found that one of our Bp35 (CD20) mAb, 1F5, induced B cells to proliferate as measured by ^{3}H-thymidine uptake, while another Bp35 mAb did not (12). We proposed that Bp35 might function as a receptor for a "second signal" distinct from the BCR, perhaps for a macrophage factor (13). In an important follow-up study, Golay and Beverley found that 1F5 anti-Bp35 (CD20) could induce resting G0 B cells to enter the G1 phase of the cell cycle (14). Similar to our results (12), they found that a BCGF could synergize with 1F5, but alone the BCGF could not activate resting B cells. Several groups had shown that two distinct kinds of signals were required for fibroblastic cells in G0 to transit through the cell cycle – a "competence" signal such as PDGF to induce cells in G0 to enter G1 and a "progression" signal such as EGF to drive cells through S phase (15, 16). Thus, we postulated that after B cells are stimulated via a Bp35 "competence" signal, they become responsive to a BCGF "progression" signal.

During 1985, we defined another set of anti-B-cell mAbs after immunizing mice with human tonsillar B cells. We enriched for B cells by depleting rosetting T cells out with sheep red cells, since magnetic beads, let alone magnetic bead kits, were not yet available. We screened the mAbs for their ability to induce B cells to proliferate and found that one of them, G28-5, dramatically increased B-cell proliferation, but only when anti-mu sera or 1F5 anti-Bp35 (CD20) was present. Importantly, G28-5 behaved like a "progression" signal in that it could augment B-cell proliferation even when added 48 h after the initial activation signal. Biochemical analyses using radioactive ^{125}I-labeled tonsillar cells showed that G28-5 detected a 50-kDa protein on B cells, which we called Bp50. We immediately thought that we most likely had discovered a receptor for either "a soluble growth factor or for a signal mediated through cell-cell contact" that induces a "progression" signal in B cells and that the factor might be made by T cells (3).

The G28-5 mAb ended up have a number of uses: Ivan Stamenkovic in Brian Seed's lab used G28-5 and expression cloning to isolate a cDNA encoding human CD40 (17). CD40 turned out to be related to the nerve growth factor receptor (NGFR), and after other similar receptors were identified, the receptor group was called the TNF receptor superfamily. It could just as easily been called the NGFR family after the first member cloned. We used the human CD40 cDNA sequence to isolate the mouse CD40 cDNA (18), and transfectants expressing CD40 were then used to characterize a number of useful anti-mouse CD40 mAbs.

CD40 mAbs were also used to characterize a signaling pathway that is both distinct from the BCR pathway and acts synergistically with BCR signaling; these studies help to stimulate investigation and understanding of TRAF protein-regulated signaling pathways (19). CD40 ligation was shown to activate CD18/CD11a-mediated adhesion to increase IL-6 expression, and together with IL-4 to induce isotype class switching to IgE, all before the ligand for CD40 was discovered (20–22). Liu et al. (23) made the important finding that G28-5 prevented B cells from dying, laying the groundwork for a role for CD40 in germinal center (GC) formation and studies of how lymphocytes are protected from cell death. Thus, many features and functions of the CD40 signaling pathway in B cells were made prior to the discovery of CD40L (24–26) and linking CD40 to "T-cell help."

WHAT DID WE GET RIGHT?

The hypothesis that B cells, like fibroblasts (15, 16), need two signals – a "competence" signal and a "progression" signal – to proliferate seems to hold true. In general, lymphocytes need at least two signals to divide and differentiate. The focus on the function of a receptor expressed on B cells, rather than using new mAbs to CD markers to subdivide lymphocytes into ever increasing species and subspecies – the cellular immunologist's perpetual distraction – clearly led to new insights. Focusing on a receptor's function is all the more relevant in the current world of immunological research, where each month brings new data underscoring the plasticity of lymphocytes, their ability to adapt and change in a range of environments (27, 28).

Looking back, I am amazed at how much others and we were able to discover simply by using a mAb to a particular receptor expressed on B cells. The CD40 mAb, not the cDNA, paved the way to understanding what CD40 is and does. In our first paper on CD40 (3), we concluded that "this work in turn may help in devising strategies *in vivo* for the control of human diseases such as B-cell malignancies, immune-deficiencies, and certain autoimmune diseases." Almost 30 years later, CD40 holds tremendous potential as a target for immunotherapeutics and vaccines.

Another important thing we got right was to send our CD40 mAb and other mAbs to whomever wanted them, usually as milligrams of purified protein without any strings attached, unless very large quantities were requested. We began this practice in the early 1980s and the number of requests steadily rose until by 1991 we were shipping out over 100 shipments of mAbs per year. We sent G28-5 to more than 100 labs, once to 11 labs on one day in 1993. This was at a time when there were few companies from which one could buy mAbs and none were selling anti-CD40; we felt it was our responsibility to get all the mAbs that we could out to those who could use them. I had learned the practice of open giving of reagents and ideas in science from Av Mitchison and Martin Raff in London. However, eventually we were tired of spending so much time and effort distributing mAbs. The companies that have taken over this task have done scientists a service, but of course instead of receiving milligrams of free mAb, we buy micrograms of conjugated mAbs at $350 or more a pop. I feel better when I am given something by a neighbor grown in her garden, instead of buying it at the store. The practice of science simply feels more personal when labs exchange gifts with each other without strings attached.

WHAT DID WE MISS OR NOT GET RIGHT?

While in Osaka in 1987 on sabbatical in Tadamitsu Kishimoto's lab, two students, Seiji Inui and Tsuneyasu Kaisho, and I used the new CD40 cDNA to express wildtype (WT) human CD40 and CD40 mutants in a mouse cell line M12. We found that residue T234 in the CD40 tail is essential for CD40 signaling regulating cell survival (29). M12 cells expressing WT CD40 (M12-CD40) responded to anti-CD40 with growth inhibition while cells expressing CD40 without its cytoplasmic tail (M12-tailless) did not. I decided that this pair would be ideal for identifying the ligand for CD40 (CD40L). But none of the various BCGF and BCDF that we tested inhibited the growth of M12–CD40 but not of the M12-tailless cells. Cosman and his colleagues at Immunex in

Seattle had set up a system where groups of cDNAs from a cDNA library were transiently transfected in Cos cells and supernatants screened for activity. We began collaborating with Immunex and tested a large number of supernatants from transfected cells for their ability to inhibit M12–CD40 cells but not M12–tailless cells. We were very excited when within a few months we identified a candidate supernatant that had the properties we were looking for. However, when the cDNA encoding the protein was sequenced, it turned out to encode for mouse IL-4. This was quite surprising not only because a mouse cDNA was picked up in a screen from a human cDNA library. How could it be that mouse IL-4 (and not human IL-4 we subsequently discovered) of all factors signaled cells expressing WT CD40 but not cells missing the CD40 tail? For more than a year, we tested everything we could get our hands on using the M12 screening assay including supernatants from stromal cells for possible "CD40L activity," all to no avail. I became disheartened, and we stopped working on the project. The Immunex team to their credit persevered and using another approach was able to discover CD40L (24). Although I had helped to initiate the search for CD40L, by focusing on the screen on M12 cell lines, I missed the chance to be actively involved in discovering it. The discoveries of CD40L, the CD40L defects in patients with hyper-IgM syndrome and subsequent studies with CD40- and CD40L-deficient mice established the key role of CD40 in T-cell-dependent B-cell responses (24–26, 30).

In our early publications, we focused on the role of CD40 on B cells, even though others and we early on had found that CD40 is expressed on epithelial cells and carcinomas (31). Ling et al. (32) in the third CD workshop in 1986 unequivocally showed that CD40 was expressed on interdigitating cells in T-cell zones, and Hart reported in 1988 that CD40 is expressed on human tonsillar dendritic cells (DCs) (33). In spite of knowing that CD40 was expressed on DCs, we did not test whether G28-5 could stimulate DCs for many years. We were simply too B-cell-centric! Only when Rainheim and Kipps (34) reported that CD40 ligation upregulates the expression of CD80 on B cells did we finally get around to testing if that was the case for DCs (35). By then, a number of groups were investigating the function of CD40 on DCs (36, 37); important discoveries followed, not the least of which was the discovery that CD40 signaling plays a vital role in programing CD8 T-cell responses (38). So, we missed a chance to define some key CD40 functions in DCs.

Nevertheless, we began to appreciate the active communication between T and B cells more fully and the links between the CD40L–CD40 signaling and CD80–CD28 signaling (39). CD4 T cells then and even today are sometimes referred to as "conductors", as the cells that "orchestrate" immune responses, a view bolstered not only by paternalistic thinking immunologists but also by the fact that the dreaded HIV-1 virus targets CD4 T cells. I thought of the program orchestrated by T cells and B cells as being based more on a constructive and mutual conversation (39) between the sometimes underappreciated "female" B cells and respectful "male" T cells, rather a program conducted by dominant CD4 T cells. Some days, it felt like the field of immunology was full of chauvinists who thought that "male" CD4 T cells were running the show and thus the only cells worth studying. Even today they are still 10 times or more NIH grants funded on CD8 T-cell memory than on B-cell memory, even though both cytotoxic T cells and antibodies play key roles in sustained protective immunity.

An effective outcome requires quality communication between the parties involved. We proposed that T and B cells communicate via "reciprocal dialogs" between equally important cells and involving the CD40L–CD40 and CD80–CD28 "phrases" or pathways that reinforce each other (39). I drew the diagram made to illustrate this point purposely as a yin-yang symbol with Ag-MHC class II peptide/TCR in the middle. Today, B cells are better appreciated in part due to the powerful efficacy of B-cell depletion therapies. An immunotherapy that combines blockade of both the CD40L–CD40 and CD80/86–CD28 pathways, a big part of the conversation, still holds tremendous promise for inducing Ag-specific tolerance.

Ironically, one of the biggest errors that we made was focusing on the G28-5 mAb at the expense of another mAb established in the same fusion, G28-8 (anti-Bgp95). This mAb turned out to bind to a novel member of the Toll-like receptor (TLR) family, CD180/RP105 (40), actually the first mammalian TLR to be cloned and compared to *Drosophila* toll (41). We were not able to isolate the CD180 cDNA by expression cloning since CD180, like its very close relative, TLR4, can only be expressed on the cell surface as a heterodimer (CD180/MD1). Anti-CD180 mAbs coupled to Ags efficiently induce a potent B-cell adjuvant signal and IgG Abs (42), and the combination of viral envelope Ag attached to anti-CD180, can induce protective immunity (unpublished data). Ironically, the at-times forgotten G28-8 mAb may turn out to be as interesting or more interesting than its G28-5 sister.

FUTURE CLINICAL PROSPECTS

A number of anti-CD40 Abs and CD40L blocking agents (anti-CD154 or CD40-Ig) are in clinical development for treatment of autoimmune diseases, transplant rejection, and cancers (43). For the treatment of autoimmune diseases and transplant rejection, the goal is to bring forward therapeutics that can block the CD40L–CD40 pathway as part of a "costimulation blockade" strategy, for example, to induce transplantation tolerance (44). Recently, an anti-CD40L engineered so it does not have Fc effector functions like binding to Fc receptors on platelets was shown to block the development in mice of a systemic lupus erythematosus-like disease (45).

CD40 is a particularly attractive oncology target. It is expressed on both lymphoid malignancies and on a range of carcinomas. Thus, anti-CD40 mAbs may help mediate cancer cell killing by effector cells. Furthermore, as seen with the M12 cell line (20, 29), CD40 ligation of some tumor cells can lead to cell death (43, 46). Humanized anti-CD40 mAbs are currently being tested in clinical trials for the treatment of non-Hodgkin's lymphomas (NHLs) and other cancers (43). The signaling pathway induced by anti-CD40, as we reported with normal B cells (3) is distinct from and synergistic with anti-CD20 mAbs like rituximab, and can promote increased lymphoma cell death (47–49). Thus, CD40-based immunotherapies may find use in conjunction with CD20-based therapies (43) for NHLs as well as with other agents (50).

Furthermore, anti-CD40 mAbs hold great promise for use as part of vaccines against cancers and infectious diseases [e.g., Ref. (51–53)]. In particular, CD40 ligation can promote the activation

of cytotoxic CD8 T cells (38), essential for effective therapeutic vaccines. Local administration and slow release of anti-CD40 may mitigate the potential adverse side effects associated with agents that strongly modulate the immune system (51, 52). Thus, it is very likely that one or more new CD40-based therapies will be in use in the coming decade.

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Identification of the IgG1 induction factor (interleukin 4)

Eva Severinson*

Department of Molecular Biosciences, The Wenner-Gren Institute, Stockholm University, Stockholm, Sweden
**Correspondence: eva.severinson@su.se*

Edited by:
Kendall A. Smith, Weill Medical College of Cornell University, USA

Reviewed by:
Kendall A. Smith, Weill Medical College of Cornell University, USA
Ignacio Sanz, University of Rochester, USA

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This is an opinion based on the paper "Cloning of cDNA encoding the murine IgG1 induction factor by a novel strategy using SP6 promoter" (1).

The mechanism by which B cells can produce classes other than IgM and IgD has interested me since the middle of the 1970s. It was initially not clear that B cells could produce more than one Ig class, whereas we now know that they first produce IgM and IgD and then – after activation – they can switch to produce IgG, IgA, or IgE. Interleukin 4 (IL-4) can regulate Ig class switching in mice to IgG1 and IgE, and in humans to some subclasses of IgG and to IgE. Other cytokines can induce activation of various Ig classes and subclasses. Below is a personal tale of how I became involved in the molecular cloning of IL-4.

When I was a PhD student in the beginning of the 1970s, many of us at the Department of Immunobiology, Karolinska Institutet were intrigued by the question of whether, and if so how, B-cells switched Ig class. In the laboratory, Göran and Erna Möller and their groups were studying *in vitro* responses to T and B-cell mitogens, using mouse spleen cells. While stimulation with T cell-dependent antigens *in vivo* led to IgM and IgG responses, T cell-independent antigens did not induce an IgG response. Likewise, B-cell mitogens *in vitro* gave rise only to an IgM response. Since, we were concerned that fetal calf serum contained mitogenic substances; we used serum-free cultures. However, in order to obtain any response at all, we had to culture cells very densely. Kearny and Lawton then published a paper, describing the low-cell-density culture system (2). By culturing spleen cells at much lower cell densities, and by including fetal calf serum and 2-mercaptoethanol together with the mitogen lipopolysaccharide (LPS), B cells were induced to produce both IgM and IgG. This was an important discovery, since it was the first time IgG production had been induced *in vitro*. We tried to reproduce the experiment, but we had no good way to measure a polyclonal IgG response. At that time, we measured IgM responses in a plaque assay, using haptenated sheep erythrocytes, but it was not suitable for measuring IgG responses. Kearny and Lawton had used intracellular fluorescence in a UV microscope to detect IgG-producing cells, but we wanted a more quantitative assay. The idea occurred to me to develop an assay using protein A. It binds IgG and thus, by coating it to sheep erythrocytes, we might be able to develop a plaque assay for IgG-secreting cells. Antonio Coutinho and I decided to try the idea, so I went to visit him at Basel Institute for Immunology in Switzerland, where he worked as a postdoc. He thawed different mouse plasmacytomas that Fritz Melchers had in his lab. We could also use rabbit antisera to different mouse Ig isotypes, also from Fritz's lab. I came to Basel in January 1976 and, if I remember correctly, the experiment worked first time. However, there were no plaques in the absence of developing antisera, as I had predicted. A likely explanation is that the rabbit IgG antisera bound the protein A and thus formed a bridge between protein A and the secreted Ig. This meant that we could detect any class of Ig from secreting cells if we just had a suitable antiserum. We worked day and night for 6 weeks to further develop the technique. More than once I met Susumi Tonegawa at the soft-drinks machine at night. He was then finishing his ground-breaking work on the movement of Ig V genes in B cell-development. A few months later, he presented his results at a Cold Spring Harbor meeting and I was in the audience. That was a very memorable moment. I left for the US 6 weeks later, and Antonio and Fritz finished the paper about the protein A plaque assay (3). Now, there was a good and convenient method to detect IgG-secreting cells.

I started as a postdoc in Sam Strober's lab in Department of Medicine, at Stanford University Medical Center, in February 1976. Upstairs from his lab, in Len and Lee Herzenberg's group at the Department of Genetics, there was intense research using one the first flow cytometry machines. I wished to determine if the IgG response obtained after LPS stimulation *in vitro* was derived from IgM-producing cells, i.e., if there was a true switching event occurring in the cultures. I sorted IgM and IgG positive and negative cells and cultured the populations separately, and could show that the IgG-producing cells came from IgM^+, IgG^- precursors (4). One curious observation was that the main IgG subclasses produced were IgG2b and IgG3, and we did not detect very much IgG1, although the latter is the dominant subclass in serum.

Back in Stockholm a few years later, I read a manuscript by Peter Isakson et al. in Ellen Vitetta's lab about a factor that induced an elevated IgG response, mostly of the IgG1 subclass, when given to LPS cultures. Their paper was published in 1982 in *J Exp Med* (5), in the same issue as a paper by Maureen Howard et al. in Bill Paul's group about a factor that induced DNA synthesis in B cells together with anti-IgM (6). These two papers are to my knowledge the first to describe the function of what later became known as interleukin 4 (IL-4). Peter used supernatants from different T cell lines. We tested supernatants from primary mixed

lymphocyte cultures that we obtained from Antonio Coutinho and they worked in a similar way, the elevated IgG being mostly IgG1. We were Susanne Bergstedt-Lindqvist, Paschalis Sideras, both PhD students and myself. Although they worked with me, neither were officially my students, because I only had a very small grant at the time.

It became clear that the stimuli dictated the subclass produced by B cells. The T-independent LPS gave rise to IgG2b and IgG3, but addition of factors produced by T cells caused B cells to produce IgG1 instead.

We realized eventually that we needed a more reliable source of the IgG1 induction factor, which was the name we gave it. For this reason, Paschalis and I and went to Lausanne to work in Marcus Nabholz's lab at ISREC (Swiss Institute for Experimental Research). This was in the spring of 1983. In the neighboring lab Rob MacDonald and his group were working with continuous T cell lines from primary cultures. From the very back of the incubator, we retrieved a flask that appeared to have been forgotten. We re-stimulated the cells, cloned them and tested the supernatants for IgG1-inducing activity. There were several clones that were positive. Rob suggested later that the fact that the flask had been forgotten might have increased the chances to select for IL-4 secreting cells, due to autocrine activation. Usually, cells were re-stimulated with irradiated stimulatory cells plus IL-2, which most would probably have repressed IL-4 production.

We took the T cell clones with us back to Stockholm and decided to characterize the IgG1 induction factor in biochemical terms. For this, we developed a quantitative assay, using serial dilutions of the factor in cultures. We obtained this idea from Kendall Smith's work with IL-2. We had met Ken at conferences and he was always very supportive of our work. We wrote a paper describing the different T cell clones, one of them being the 2.19 cell line, which we used later on to clone IL-4 (7). Another paper of ours described the biochemical characteristics of the IgG1 induction factor, which indicated that our factor had properties similar to Paul's B-cell-stimulating factor (8). When Paschalis showed our data at a Keystone meeting, it did not attract very much attention. The general idea at the time was that each function was regulated by distinct factors and thus it was considered very unlikely that the same factor would induce proliferation together with anti-IgM and an elevated IgG1 response together with LPS.

In August 1983, I went to the Congress of Immunology in Kyoto. It was terribly hot and I was 7 months pregnant. The most exciting talks were those by Ellis Reinherz, Mark Davis, and James Allison, describing for the first time the T cell receptor. Also, I remember several people being very upset with the scientists who had claimed that T cells expressed immunoglobulin. That hypothesis died completely at this conference.

In connection with this meeting I visited Tasuku Honjo, who was then working at Osaka University. I had met him twice before in Sweden. He was well known for having correctly determined the gene order of the heavy-chain C gene segments in mice and for discovering the heavy-chain switch regions (9). I gave a seminar about our preliminary data with the IgG1 induction factor. Tasuku suggested collaboration: "I would like to clone switch factors" he said. Back in Sweden in December of 1983, I gave birth to my first child, John.

To attempt to clone the IgG1 induction factor without having purified it or a specific antibody was a bold attempt, which I had thought would not be within my reach. Tasuku, being a careful person and probably a bit suspicious of the cellular techniques, wanted first to test our assay to measure the response. So we sent him the protocol together with the necessary ingredients and they obtained the same response as we did. Paschalis and Susanne then started to collect 10^9 cells of the 2.19 cell line to send to Honjo's group. This took several months, since these were primary T cells stimulated by irradiated allogeneic spleen cells and they grew rather slowly. The final stimulation was done with concanavalin A (ConA). We sent the cells by the end of June 1984 and people in Honjo's group prepared mRNA. A second batch of cells was sent later. At this time, we also received a lot of help from Lena Berggren (now Lena Ström), our technician. She eventually got a PhD with me as her supervisor and is now an established scientist working with genome stability and variation. Honjo's group purified mRNA and as a first test, injected it into *Xenopus* oocytes and collected supernatants. The supernatants indeed gave an increased IgG1 response when given to LPS cultures. This result was obtained in October 1984. Then, Honjo's coworkers transfected COS cells with a cDNA library from the 2.19 mRNA, took supernatants and sent them to us in Sweden. However, COS cell supernatants were inhibitory when given to LPS cultures, perhaps due to mycoplasma-infection, since this notoriously inhibits B cell activation *in vitro*. Tasuku was compelled to change strategy, and came up with a very ingenious one: They constructed an Sp6 expression vector and cloned cDNA out of 2.19 mRNA, did *in vitro* transcription, and injected the RNA into oocytes. The oocyte supernatants were then sent to us for testing. This strategy worked well and we could detect IgG1-inducing activity for as many as 45,000 mixed clones. The numbers of clones per batch decreased for each round of testing, until we finally had single positive clones. We reached single clones in the summer of 1985. The cDNA from a positive clone was sequenced and found to encode a protein of MW around 15,000. It had some homologies suggesting a distant relation to γ interferon (γIFN) and granulocyte macrophage colony-stimulating factor (GM-CSF). We tested the recombinant supernatant and found that it induced higher MCH class II levels and increased DNA synthesis when given together with anti-IgM. We concluded that the IgG1 induction factor and the B-cell-stimulating factor-1 (BSF-1) were products of the same gene. First, we wrote two papers (letters) that we submitted to Nature. One, for which Yoshihiko Noma was first author and Tasuku was senior author, dealt with the molecular cloning. The other, for which Paschalis was first author and I was senior author, was about the function of the cloned factor on B cell responses. The editors of Nature wished us to merge the two papers and invited us to write an article instead. This we did, and the paper was published in February 1986 (1). In April the same year, Lee et al. published a paper identifying the same cytokine, also by cDNA cloning. The cytokine could activate T and B cells, as well as mast cells. It acted as a B-cell-stimulating factor, and induced IgG1 and IgE (10). At the end of both of the papers, we proposed that the cytokine should be called "interleukin-4." Our paper

attracted a lot of attention, partly because the cloning method was unique. It should be pointed out that cDNA cloning at this time was not a technique that any lab could do. In fact, only a few groups in the world mastered it, and Honjo's lab was one of the best. Secondly, the two papers revealed that IL-4 was very pleiotropic, having effects on at least three cell types and inducing different responses in B cells, as opposed to other known cytokines, and quite different from the idea that one cytokine would only activate a single response. The molecular cloning of IL-4 inspired a great deal of further research. In August the same year, a paper was published about cloning of the cDNA that encoded human IL-4 (11). Soon it became apparent that both human and mouse IL-4 induced an IgE response (10, 12) and it was subsequently showed, using the knockout technology, that IL-4 is essential for IgE expression in mice (13). Thus, IL-4 was found to be clinically relevant for understanding the induction of atopic allergy. Furthermore, the finding that IL-4 is secreted by a subpopulation of T helper cells, called TH2 (14), inspired a flood of research in humans. In the field of Ig class switching, IL-4 is still one of the model stimuli in research of class switch recombination in the mouse, because of its efficient induction of IgG1 and because the discovery that stimuli direct switching by inducing germ-line transcripts (15, 16). Many cytokines have been described since then: a glance at internet reveal the existence of IL-38.

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Revisiting the identification and cDNA cloning of T cell-replacing factor/interleukin-5

Kiyoshi Takatsu [1,2] *

[1] *Toyama Prefectural Institute for Pharmaceutical Research, Imizu City, Toyama, Japan*
[2] *Department of Immunobiology and Pharmacological Genetics, Graduate School of Medicine and Pharmaceutical Sciences, Toyama City, Toyama, Japan*

Edited by:
Kendall A. Smith, Weill Medical College of Cornell University, USA

Reviewed by:
Michael Croft, La Jolla Institute for Allergy and Immunology, USA
John William Schrader, The University of British Columbia, Canada

****Correspondence:***
Kiyoshi Takatsu, Toyama City, Japan
e-mail: kiyoshi.takastu@pref.toyama.lg.jp, takatsuk@med.u-toyama.ac.jp

This is a perspective based on the paper "*Cloning of complementary DNA encoding T cell-replacing factor and identity with B cell growth factor II*," by Kinashi et al. (1). We have been interested in understanding the molecular basis of T–B cell cooperation for antibody formation. Although many investigators had described a number of different soluble factors that appeared to have biological relevance to T–B cell interactions, molecular basis of such active substances remained unknown for a long period of time. In this perspective, I will briefly summarize the history of the initial discovery of T cell-replacing factor/B cell growth factor II that appeared to be involved in B cell growth and differentiation, and outline the discovery and characterization of interleukin-5. Studies of interleukin-5 have provided strong evidence that a single cytokine exerts a variety of activities on diverse target cells.

Keywords: B cell differentiation factor, eosinophil differentiation factor, B cell growth factor, TRF, IL-5

The reciprocal relationship between antibody production and cell-mediated immunity had been well established by 1970. Antigen doses, forms, and routs of administration for stimulating optimum antibody production to T-dependent antigens were found to be usually accompanied by little or no delayed-type hypersensitivity (DTH), and vice versa, despite the fact that both B cell help and DTH were properties of T cells (2). Thus, one of the key issues was how antigen-stimulated T cells regulate both humoral and cell-mediated immune responses. The other issue was how T cells helped B cell differentiation into antibody-secreting cells.

The articles of Rajewsky et al. (3) and Mitchison (4) described the involvement of the cellular cooperation of carrier-specific T cells and hapten-specific B cells for anti-hapten antibody response to hapten-carrier conjugates. Besides the carrier specificity of helper T cells, these cells also strictly recognized MHC class II molecules on antigen-presenting cells. However, in some cases, the interactions between helper T and B cells were not restricted by MHC class II. This was explained by the possibility that both T and B cell populations were functionally heterogeneous. The mechanism underlying B cell triggering in the latter case was interpreted to be mediated by a T cell helper effect via the release of a soluble factor.

Evidence for the existence of such soluble factors came initially from the demonstration in the pioneering studies of Dutton et al. (5), and Schimpl and Wecker (6), both of whom observed that the antibody response of T cell-depleted mouse B cells to the antigens of sheep red blood cells (SRBC) required T cell-derived and macrophage-derived soluble factors detectable in the supernatants obtained from short-term cultures of histoincompatible mouse spleen cells or Concanavalin A stimulation of mouse spleen cells. Although various investigators had described a number of different factors that appeared to have biological relevance to T–B cell interactions, which T cell population secreted such active substances remained unknown. In other words, it had not been established whether those helper T cells that released biologically active mediators belonged to the same T cell population that collaborated with B cells in direct cell-to-cell contact (cognate interactions), or whether these two activities were due to two distinct T cell subsets.

In this review, I will briefly summarize the history of the initial discovery of an activity that appeared to be involved in B cell growth and differentiation, and outline the developments that led to the discovery and characterization of the molecule that was designated interleukin-5 (IL-5).

We had been interested in the adjuvant effects of *Mycobacterium tuberculosis* (Tbc) on both humoral and cell-mediated immune responses. In the early 1970s, we focused on two questions regarding T and B cell cooperation for antibody production: whether a helper T cell subset for B cell activation was also responsible for DTH as effector T cells, and whether T cell-derived factors were involved in B cell activation in the absence of cognate T cell help for antibody production. At that time, the existence of T cell-derived factors that act on B cells in an antigen-non-specific manner was not appreciated.

Since Tbc was well known as an important ingredient of Freund's complete adjuvant, widely used to enhance antibody production and also to augment DTH responses to a co-immunized particular antigen, we examined tuberculin (PPD)-reactive T cell activity in spleen cells from heat-killed Tbc-primed mice. Helper T cell activity of Tbc-primed cells was assessed by an adoptive cell transfer system in mice by the ability to induce anti-2,4-dinitrophenyl (DNP) IgG antibody-secreting cells from DNP/keyhole limpet hemocyanin (DNP-KLH)-primed mouse B cells. As expected, anti-DNP IgG antibody production was induced by stimulating DNP-primed B cells with DNP-PPD in the presence of Tbc-primed cells (7). However, significant anti-DNP IgG antibody production was also enhanced by stimulating DNP-primed B cells with a DNP-coupled heterologous carrier plus PPD, but

only when Tbc-primed cells were present. Furthermore, injection of conditioned medium of PPD-stimulated Tbc-primed cells into recipient mice with DNP-primed B cells augmented the anti-DNP IgG response compared with control supernatants. We tentatively called the active factors produced by T cells as factors that enhanced anti-hapten antibody production.

These findings implied that Tbc immunization of mice could prime two types of helper T cells regarding B cell help; one triggered B cells through direct cell-to-cell contact via the DNP-PPD (cognate interaction) and the other activated their indirect interaction via lymphokine secretion (factor-mediated interaction). We evaluated these possibilities by an *in vitro* culture system, the results of which revealed that Tbc-primed T cells for cognate interaction (Thc) developed as early as 7 days after Tbc-priming and collaborated with T cell-depleted DNP-primed B cells upon stimulation with DNP-PPD. However, Tbc-primed T cells for factor-mediated interaction (Thf) only developed after 4 weeks of priming and could augment the B cell response only in the presence of PPD (8). We further confirmed the existence of two distinct types of helper T cell subpopulations, representative of Thc and Thf, in Tbc-primed cells by establishing two distinctly functioning, long-term-cultured PPD-reactive helper T cell clones. Supernatants of PPD-stimulated Tbc-primed cells enhanced anti-DNP IgG production by DNP-primed B cells in an MHC-non-restricted manner (9). We referred to the enhancing factor(s) as T cell-replacing factor (TRF) because the assay systems for and biological properties of the enhancing factor(s) thus described were similar to those reported by Schimpl and Wecker in 1972 (6). We also found a strain of mice, DBA/2Ha, whose DNP-primed B cells had an X-linked B cell defect, reflected in part by the low responsiveness to TRF, and were unable to be activated through factor-mediated interaction, while they were good responders with Tbc-primed T cells for cognate interaction through DNP-PPD, suggesting the existence of two different subsets in activated B cells (9).

To obtain further insight on the molecular basis of TRF, we took two different approaches. First, we established a monoclonal T cell hybrid clone B151K12 by means of the fusion between Tbc-primed BALB/c T cells and BW 5147 thymoma (10). B151K12, which we referred to as simply B151, produced TRF-active molecules continuously, without stimulation. The cell-free supernatant of B151 could induce an anti-DNP IgG-PFC response in T cell-depleted DNP-primed B cells from various strains of mice. It also restored the primary anti-SRBC IgM PFC response in *nu/nu* spleen cells. As B151 did not produce detectable levels of other cytokines affecting B cell responses, we hypothesized that this B151-derived TRF (B151-TRF) was a novel cytokine, distinct from other cytokines. B151-TRF was able to augment B cell responses in a totally antigen-non-specific manner and in MHC non-restricted manner.

Secondly, we selected a particular clone of *in vivo* growing murine chronic B leukemia (BCL1) cells that preferentially responded to B151-TRF and LPS, and differentiate into polyclonal IgM-secreting cells. BCL_1 cells appeared to be good target cells for B151-TRF and thus we adopted them for further analysis of TRF-active molecules.

Once B cells are activated, there is an early phase of proliferation in which the responding B cell population expands many times, and a later phase of differentiation induced by TRF in which the already proliferating B cells move on to become Ig-secreting cells. In the early 1980s, several interesting observations were reported indicating that different phases of the B cell response to antigenic stimulation are regulated by functionally and biochemically distinct factors. Howard et al. demonstrated a B cell growth factor (BCGF) in supernatants of PMA-stimulated thymoma cells (designated EL-4) that co-stimulated purified B cells with anti-IgM antibodies in short-term cultures, and induced polyclonal B cell proliferation but not antibody-forming cell generation (11). The BCGF was not mitogenic for resting B cells and interacted with anti-IgM-activated B cells in a non-H-2-restricted manner. The factor was distinct from T cell growth factor (IL-2) and synergized with antigen, IL-2, and IL-2-free, BCGF-free T cell supernatants that contained TRF to generate antibody-forming cells in cultures of highly purified B cells (11). This was the first description of a lymphokine activity (distinct from IL-2) that had the ability to support B cell proliferation, and was tentatively called BCGF (later referred to as BSF-1 and after molecular characterization, IL-4), and the activity eluted at an apparent m.w. of 18 kDa on gel filtration. BCGF(IL-4) was able to synergize with two additional TRF activities to trigger B cells to Ig-secreting cells; one was a TRF-containing cell-free supernatant from B151, and the other was a TRF activity derived from PMA-stimulated EL-4 cells. However, it was not clear whether these two TRF activities were identical or distinct.

Concomitantly, Swain and Dutton reported a second BCGF with an apparent m.w. of 50 kDa on gel filtration in a cell-free supernatant from an IL-2-dependent T cell line (designated Dennert) that induced the proliferation of dextran sulfate-stimulated B cells or BCL_1 cells, whereas it did not induce anti-IgM-stimulated B cells to proliferate (12). The 18 kDa BCGF described by Howard et al. was designated as BCGFI and the 50 kDa BCGF as BCGFII. Soon after that, Swain found that their BCGFII preparation was able to induce Ig-secretion from activated B cells and that this differentiation activity co-purified with the proliferative activity in a variety of chromatographic separations. We demonstrated that B151-TRF could induce not only differentiation but also the proliferation of B cell populations that were co-stimulated with anti-IgM antibody plus BCGFI. Swain and Dutton independently demonstrated that B151-TRF promoted the proliferation of DXS-stimulated B cells or BCL_1 cells, suggesting that B151-TRF exerted BCGFII activity on pre-activated B cells. An important question became whether B151 cells produced both TRF and BCGFII or whether a B151-TRF molecule also exerted BCGFII activity.

When we started to purify TRF-active molecules to homogeneity, we were in the front line among TRF-hunting groups. However, concepts of BCGFI and BCGFII for B cell growth and differentiation had by this time become accepted by most workers in the field. So, we had to clarify whether B151-TRF was identical to either BCGFI or BCGFII. We examined the BCGFI activity of our purified B151-TRF using anti-IgM-stimulated B cells and found that B151-TRF did not show detectable levels of BCGFI activity. However, we independently confirmed the activity of B151-TRF on BCL_1 cells, thereby defining that it had BCGFII activity (13).

Our strategy for identifying TRF(BCGFII) was to first purify TRF-active molecules to homogeneity from the cell-free

supernatant of B151, including the identification of its partial NH2-terminal amino-acid sequence. Overall, B151-TRF was purified approximately 1,400,000-fold, and the activity could be attributable to an extremely hydrophobic glycoprotein with a molecular mass of 40–60 kDa and a smaller mass (25–30 kDa) under reducing conditions on gel filtration. Highly purified B151-TRF also showed BCGFII activity, whereas it did not exert detectable IL-1, IL-2, IL-3, (BCGFI)IL-4, or IFN-γ activities. During each purification step, the BCGFII activity could not be separated from the TRF activity and always resided in the same fraction in which the TRF activity was detected (13).

Although we attempted to determine the partial NH2-terminal amino-acid sequence of purified B151-TRF several times, we were unsuccessful. So, we immunized rats with purified B151-TRF and their spleen cells were fused with mouse myeloma cells to generate monoclonal antibodies (mAb) reactive with B151-TRF. We obtained two different anti-mouse TRF mAb, designated as NC17 and TB13, which neutralized the TRF and BCGFII activities of B151-TRF, and blocked B151-TRF-induced IgM secretion and the proliferation of BCL_1, whereas they did not inhibit activities of IL-1, IL-2, IL-3, (BCGFI)IL-4, and IFN-γ (14). Immobilized mAb TB13 as well as NC17 proved feasible for reproducibly purifying TRF-active molecules from the conditioned medium of B151 and PPD-stimulated Tbc-primed cells with molecular weights of approximately 50 kDa under non-reducing conditions (15).

To obtain concrete evidence that the molecular properties of mouse TRF and BCGFII were in fact identical, we isolated a cDNA encoding TRF from a 2.19 T cell line by using a pSP6K cDNA library, in collaboration with Honjo's group. Since structural characterization was not available at that time, we felt that the best strategy was to use an expression vector system that required only a limited amount of mRNA. We decided to apply and construct a new expression vector system, pSP6K containing the SP6 promoter (16). With the aid of a specific RNA polymerase, the pSP6K vector allowed the synthesis of up to a few micrograms of poly A^+ RNA by *in vitro* transcription. This mRNA was directly injected into *Xenopus* oocytes and the oocyte culture supernatants were analyzed for their biological activities. In this way, secretory proteins could be synthesized in a system that was mycoplasma-free, free of serum proteins, and highly concentrated.

As cultured supernatants of the 2.19 T cell line exerted TRF activity like B151-TRF, poly A^+ RNA of 2.19 T cells was fractionated and enriched by sucrose gradient fractionation and aliquots of RNA were microinjected into *Xenopus* oocytes and the oocytes' culture supernatants were analyzed for TRF and BCGFII activities (1). cDNA libraries from polyA^+ RNA and from the mRNA enriched by the sucrose gradient fractionation were constructed using the pSP6K vector system. Pools that scored positive in the biological assays were further divided into smaller pools that were analyzed in the same manner until single cDNA clones capable of directing the synthesis of biologically active TRF preparations were obtained. Finally, pSP6K-mTRF was shown to encode TRF activity (1). The isolated cDNA clones were then subjected to nucleotide sequencing analysis.

We obtained a cDNA clone, pSP6-mTRF23, which after hybrid selection of mRNA, a translation product was obtained that showed both TRF and BCGFII activities (1). Both TB13 and NC17 mAbs neutralized TRF and BCGFII activities of products secreted by *Xenopus oocytes* that had been injected with mouse TRF mRNA transcribed from plasmid pSP6K-mTRF23 (17). The primary sequence of TRF deduced from the cDNA revealed a novel interleukin-type molecule consisting of 133 amino acids. The putative secreted core polypeptide has a relative m.w. of 12.3 kDa that is close to that of B151-TRF (18 kDa) after glycosylation. Recombinant TRF was also found to be a dimer; reduced and alkylated recombinant TRF lost both TRF and BCGFII activities. These properties of recombinant TRF agreed with the criteria derived from B151 cells. We determined the partial NH2-terminal amino-acid sequence of 13-residues of immunoaffinity-purified recombinant TRF and B151-TRF, and found that they were nearly identical (17). A single amino-acid sequence of each sample obtained beginning from methionine was identical to that predicted from our cDNA, supporting the notion that secreted TRF polypeptide consists of 113 amino acids.

Although TRF was initially believed to be principally active on B cells, recombinant TRF was shown to increase the expression of IL-2 receptor on antigen-stimulated thymocytes, inducing them into cytotoxic T lymphocytes (CTL) in the presence of IL-2. TRF also induced the terminal differentiation of hematopoietic progenitor cells into eosinophils, besides the activities on activated B cells (17). As TRF was the fifth interleukin activity for which the primary structure had been determined, we proposed that TRF and BCGFII be called interleukin-5 (IL-5) (1). The molecular cloning of cDNA encoding both murine and human IL-5 convincingly demonstrated that a single molecule was responsible for both TRF and BCGFII activities. However, in humans, the major target cells for IL-5 are eosinophil myeloid progenitors and mature eosinophils. Human peripheral blood B cells do not respond to human IL-5 by differentiation to Ig-secreting cells. Consequently, the human B cell growth and differentiation interleukins for peripheral blood B cells appeared to be IL-2, IL-4, and IL-6 (18, 19). Subsequently, as time progressed, several new cytokine molecules would be described that influence B cells in addition to these first three discovered.

Studies on IL-5 have provided strong evidence that a single lymphokine exerts a variety of activities on diverse target cells. Together with similar properties of IL-4, these results helped many immunologists escape earlier beliefs that one lymphokine is required for each activity and that growth factor activity and differentiation factor activity are necessarily different molecular entities.

I focused on the historical perspective of the discovery of T cell derived IL-5 that promoted B cell growth or differentiation. Around the same time, largely between 1985 and 1987, there were parallel efforts by a number of groups working on eosinophil differentiation [e.g., Ref. (20)] and T cell factors that enhanced IgA production [e.g., Ref. (21)] that ultimately also led to purification and identification of IL-5.

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Revisiting the 1986 molecular cloning of interleukin 6

Toshio Hirano*

Osaka University, Suita, Osaka, Japan
**Correspondence: hirano@molonc.med.osaka-u.ac.jp*

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Kendall A. Smith, Weill Medical College of Cornell University, USA

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We reported molecular cloning of B cell stimulatory factor-2 (BSF-2)/interleukin 6 (IL-6) in 1986 (1), the same year that IL-4 and IL-5 were cloned (2, 3). Prior to the publication of our article (1), it was known that antigenic stimulation induced growth and differentiation of B lymphocytes into antibody forming plasma cells with the help of T lymphocytes and this function of T lymphocytes could be replaced by soluble factors. Dutton in 1971 and Schimpl and Wecker in 1972 reported the presence of soluble factors that induced immunoglobulin production in B lymphocytes in the absence of T lymphocytes (4, 5). Schimpl and Wecker named the putative factor "T cell replacing factor" (TRF). In addition, Kishimoto and Ishizaka reported a soluble factor that enhanced IgE antibody production (6), while Takatsu and his colleagues reported a factor that enhanced anti-hapten antibody production (7). The molecular characteristics of these soluble factors, however, were unknown. Furthermore, other reports showed the possibility that there might be several kinds of soluble factors that affected the biological activities of B lymphocytes differently (8, 9). For example, Paul and his colleagues showed that the culture supernatant of mouse thymoma EL4 in combination with submitogenic doses of anti-IgM antibodies stimulated the proliferation of B lymphocytes without inducing antibody production (10). The putative factor was called "BCGF-I" or "BSF-1." Swain and Dutton showed that culture supernatants from a long-term alloreactive T cell line, DL, induced growth in dextran sulfate stimulated B lymphocytes and BCL1 B cell tumors. The same culture supernatant when combined with IL-2 induced another response: antibody production in B cells (11). This putative factor was called "BCGF-II." Thus, prior to our article, little was known about the molecular nature of the factors responsible for B lymphocyte stimulation, which is why many immunologists reported each factor by a different name based on its biological activity.

After studying at A. Nordin's lab in the United States National Institutes of Health from 1973 to 1976, I returned to Y. Yamamura's lab at Osaka University Medical School, where we showed that PWM-stimulated human T lymphocytes produced soluble factor(s) that induced immunoglobulin production in human B lymphocytes (12). In 1978, I moved to Osaka Prefectural Habikino Hospital where I saw many patients with tuberculous pleurisy. What I found there was that purified protein derivative (PPD)-stimulated pleural effusion cells from these patients produced soluble factor(s) that induced immunoglobulin production in PWM-stimulated human B lymphocytes (13). This finding led me to start purifying the putative factor in 1978. In 1981, Kishimoto's group showed the presence of a soluble factor that enhanced IgG production in an Epstein–Barr virus transformed human B cell line, CESS. This putative factor was named "TRF" (14). We showed that the culture supernatant of PPD-stimulated pleural effusion cells obtained from patients with pulmonary tuberculosis or PWM-stimulated tonsillar mononuclear cells induced IgG production in a variety of Epstein–Barr virus-transformed B lymphoblastoid cell lines (15), which we called a "TRF-like factor" or "B cell differentiation factor II" (BCDFII) (15–17). We showed that the TRF-like factor/BCDFII was recovered in the fractions corresponding to the molecular weights 22 and 36 kDa by gel filtration, and that it had an isoelectric point between 5 and 6 (15).

In early 1984, I joined Kishimoto's lab as an Associate Professor at Osaka University and continued purifying the putative factor from the culture supernatant of the HTLV-transformed human T cell line, TCL-NA1. We soon thereafter determined the sequence of its 14 N-terminal amino acids with the help of S. Tsunasawa, Institute for Protein Research, Osaka University at the end of 1984. The purified material had molecular weights of 19 and 21 kDa as identified by NaDodSO4/PAGE under reduced as well as non-reduced conditions. The IgG-inducing activity was found in the fractions corresponding to the molecular weights noted above and the isoelectric point ranged from 5.0 to 5.1 (18). We concluded that this material, which we called "BCDF" or "BSFp-2," had properties what were similar to those of the TRF-like factor/BCDFII (15). Importantly, the purified protein did not exhibit any of the activities of IL-1 or -2, BCGF-I/BSF-1, BCGF-II or interferon (18).

Then we started the molecular cloning of the purified protein, using a probe based on information about the N-terminal partial amino-acid sequence with the kind help of T. Taniguchi, Osaka University. However, this procedure was much harder than I expected. Because of the many false positive clones bound to the probe, we were unable to obtain the cDNA encoding of the molecule with the identified N-terminal amino-acid sequence in 1985. This caused us great consternation, and we worried whether the identified sequence was correct. During this period, I suffered severe arrhythmia, which I attribute to the stress of the project. At the very beginning of 1986, I decided

to purify the protein using newly obtained culture supernatants from TCL-NA1 cells. This time, instead of obtaining the N-terminal partial amino-acid sequence of the purified protein, I decided to acquire several fragments of the purified protein by digesting the protein with lysylendopeptidase, following the thoughtful advice of S. Tsunasawa. There was a serious risk of losing all the purified protein, however, due to the additional process of separating the fragmented peptides with high performance liquid chromatography. Nonetheless, our previous failures convinced me, we had little choice. On February 20 of that year, Honjo and his colleagues reported the molecular cloning of murine IgG1 induction factor, or IL-4 (2). However, I remained firm in the need to isolate the cDNA encoding of our purified protein. We were lucky to have obtained several protein fragments during the additional purification step, which eventually led us to successfully obtain their partial amino-acid sequences by the end of March 1986. We selected three highly reliable N-terminal partial amino-acid sequences from among eight fragments to make synthetic oligonucleotide probes, which we then used to clone the cDNA. On Sunday morning of May 25, 1986, I finally found one cDNA clone out of about 30,000 that miraculously – at least in my opinion – was bound by all three probes. Thus, after 8 years of hard work, beginning in 1978 when I started the purification of helper T cell factors at Osaka Prefectural Habikino Hospital, I had finally obtained the cDNA encoding of the protein inducing immunoglobulin in B cells, which we called "BSF-2" at that time. From the cDNA sequence, we predicted that the mature form of BSF-2 is composed of 184 amino acids and has an isoelectric point of 5, again, very similar to the TRF-like factor/BCDFII (15). This result was published in a November issue of *Nature* in 1986 (1) together with a report on the molecular cloning of IL-5 (3). The sequence of BSF-2 was found to be identical to that of an IL-1-induced 26-kDa protein with unknown biological activity, which was reported in the September issue of the *European Journal of Biochemistry* (19) and the sequence of IFN-β2, which was reported in the October issue of the *EMBO Journal* (20). In addition, the plasmacytoma/hybridoma/myeloma growth factor and the hepatocyte-stimulating factor were found to be identical to BSF-2 (21–25). At a nomenclature meeting in New York chaired by W. E. Paul at the end of 1988, the community agreed to call this molecule "IL-6" (26).

Our project originally began with the desire to isolate a factor responsible for stimulating B lymphocytes to produce immunoglobulin. What we found was something far more complex. IL-6 is a multifunctional cytokine that plays roles in immune responses, inflammation, hematopoiesis, and the endocrine and nervous systems, even though it has no interferon activity (27–29). Additionally, we revealed that IL-6 might be involved in autoimmune diseases and inflammatory diseases (30–33), opening the way for new therapies that regulate inflammatory diseases (34–37).

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Deciphering thymic development

Harald Von Boehmer [1,2] *

[1] Department of Microbiology and Immunobiology, Harvard Medical School, Boston, MA, USA
[2] Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute, Boston, MA, USA
*Correspondence: harald_von_boehmer@dfci.harvard.edu

Edited by:
Kendall A. Smith, Weill Medical College of Cornell University, USA

Reviewed by:
Herman Waldmann, University of Oxford, UK

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In 1979, some of us were surprised by the (1) conclusion of Doherty and Zinkernagel on MHC-restricted antigen recognition following the lead of Katz, Hamaoka, and Benacerraf (2) describing the same for the interaction of T helper cells with B cells as well as Rosenthal and Shevach (3) describing it for the interaction of T cells with macrophages. Since Doherty and Zinkernagel offered the least complicated system, they got most of the credit. We wanted to know whether a single effector cell was involved and therefore analyzed a clone of cells, specific for the HY transplantation antigen. If it could be shown that the progeny of a single cell was MHC restricted, we had something to explain, which was not obvious. The cloning in Basel worked fine, with the competent help of Hans Hengartner before his departure to the lab of Zinkernagel in Zurich. The clone was in fact MHC restricted, telling us that MHC restriction was the property of a single cell. The same conclusion was derived from experiments with Matthias Wabl, who observed the killing of targets by single killer cell (4). The clone was also alloreactive, which was observed prior to the realization that a significant portion of T cells carried two receptors and thus it is unclear to date whether a second receptor was involved or not. This was just the first example of a clone, which was MHC restricted and HY specific as well as H-2Dd specific and this overlap in specificity was subsequently observed in other clones such that the high frequency of alloreactive T cells is not really an issue.

Follow up experiments with the Michael Steinmetz lab transferring TCR alfa and beta genes from one T cell clone to another allowed us to unequivocally conclude that the MHC-restricted specificity was encoded by a single receptor long before crystallographic studies reached the same conclusion (5). This surprised some molecular biologists somewhat who thought that the cloning of the TCR put an end to the mysteries of the immune system.

This lead automatically to the next step, the construction of TCR transgenic mice, to analyze the selection of T cells according to their specificity. Initially, we were interested to test the ideas of Burnet and Lederberg that autoaggressive cells were eliminated in primary lymphoid organs. For this reason, we used again the genes of HY specific clones since then we could easily compare female and male mice. Here, I have to tell a little tale that characterizes (some?) scientists: it was Michael Steinmetz, who had previously spoken to Fritz Melchers, who asked at the Reisensburg in the South of Germany whether there would be any interest in generating TCR transgenic mice. I answered with a clear yes saying that this would allow to test Burnets and Lederbergs ideas. So, it was concluded to go ahead and initially Georges Koehler was singled out as the scientist residing by now in Freiburg to help with the construction of mice since he had succeeded to generate immunoglobulin transgenic mice. I was therefore mildly surprised when one day Georges entered my lab and asked me whether I could give him an HY specific clone since he had the idea of testing Burnets and Lederbergs ideas. I told him that this sounded familiar, he blushed only a little and then asked Hans Georg Rammensee who was in the same office a related question. I leave it to the audience to imagine what Michael Steinmetz told Georges Koehler or better what he did not tell him, even though one cannot be completely sure of it. So Georges did not produce the mice but Anton Berns in Amsterdam cooperated and very nicely mapped what was required to express TCR beta genes in transgenic mice. Finally, the co-injection of alfa and beta genes from an HY specific clone was done by Horst Bluethmann at Hoffmann La Roche in Basel where Michael Steinmetz had moved. When the mice had grown up, we tested them with a variety of reagents prepared for this task and could report on the deletion of $CD4^+8^+$ cells in male mice even though these mice came with an anomaly, the too early expression of the transgenic TCR, which made proper quantitation difficult (6). Only recently could we address this problem and reported deletion of $CD4^+8^+$ thymocytes in the absence of TCR editing (7). This ended a long story on the deletion of autoaggressive cells at a certain stage of development, something that had not been addressed in mice expressing superantigen specific receptors, which somewhat compromised our transgenic approach since they were conducted later and yielded results earlier albeit with the limitation that the conclusions had to be restricted to superantigens (8) whereas we dealt with conventional antigens for T cells.

The next step was related to positive selection and the matching of specificity and function. Here, the first realization was that a receptor derived from a $CD8^+$ cell

would only be expressed on $CD8^+$ cells in the transgenic mice (9, 10). The second was that there was in fact positive selection as mice with inappropriate MHC antigens not restricting the specificity of the cell from which receptor genes were obtained, failed to generate single positive cells and thus development was arrested at the $CD4^+8^+$ stage where cells died (9). This was then named death from neglect as opposed to death by negative selection which eliminated likewise $CD4^+8^+$ cells, at least when the receptor was derived from $CD8^+$ cells (7). It was then clear that it was the MHC molecules expressed in the thymus and the TCR specificity, which determined positive selection, which also led to the matching of specificity and function (10), such that $CD8^+$ killer cells were generated from immature cells expressing a class I restricted TCR (11) and as shown later $CD4^+$ helper cells were generated from immature cells expressing a class II restricted TCR and thus in other words helper cells recognized as a rule peptides entering the target cell from the outside whereas killer cells recognized peptides produced in the target cell itself. This relates to the different modes of peptide loading by class I and class II MHC antigens (12–16).

In the meantime, the molecular details of this matching process have been worked out mostly by the work of Dietmar Kappes (17) as well as Dan Littman (18) who identified transcription factors guiding this process in dependence of the signaling by the receptor expressed by immature cells. Thus, at present we have a fairly complete picture of positive selection as far as the selectable T cells are concerned while still we know relatively little about the TCR ligands that are responsible for positive selection. Here, one wonders whether thymus-specific proteasome subunits play an essential role (19). Thus, there are still some secrets in T cell development even after decades of the identification of the TCR (20).

The curiosity in T cell development is still very much alive even after retirement but I trust that the remaining issues are in good hands of younger scientific colleagues who identify the outstanding questions and think of clever experiments to address them.

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Generation of human B-cell lines dependent on CD40-ligation and interleukin-4

Jacques Banchereau [1,2] *

[1] *Jackson Laboratory for Genomic Medicine, Farmington, CT, USA*
[2] *Vaccine Research Institute, U955, Hopital Henri Mondor, Creteil, France*
**Correspondence: jacques.banchereau@jax.org*

Edited by:
Kendall Arthur Smith, Weill Medical College of Cornell University, USA

Reviewed by:
Michael R. Gold, The University of British Columbia, Canada
Edward A. Clark, University of California Los Angeles, USA

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WHERE WE STARTED

The description of factor-dependent cytotoxic T-cell lines in the late 1970s transformed T-cell biology (1). Among other events, it led to the cloning of a cDNA encoding IL-2 (2). It also led to the identification of T-cell subsets and formulation of the Th1/Th2 concept in the 80s (3). However, comparable advances in B-cell biology were lacking, partly because of the lack of availability of factor-dependent B-cell lines. This was the case despite the fact that B-cell-specific trophic factors, including BSF (B-cell stimulation Factor), BCGF (B-cell growth factor), and BCDF (B-cell differentiation factor) had been described in the supernatants of activated T cells.

The cloning at DNAX, our sister institute acquired by Schering–Plough, of a cDNA encoding BSF-1, later renamed IL-4, in mouse (4) and in human (5) was a first step forward to the definition of the molecules controlling B-cell growth and differentiation. In our laboratory, based in Dardilly near Lyon (France), we found that cultured purified human B-cells triggered with anti-B-cell receptor (BCR) and IL-4 resulted in significant B-cell proliferation as measured by tritiated thymidine counts, a common way of measuring B-cell proliferation in the 1980–1990s (6). These cultures yielded more B-cells than did naïve cultures or those exposed to anti-BCR alone or IL-4 alone. Yet, these cultures established with anti-BCR plus IL-4 yielded less viable B-cells than were input. Thus, we, B-cell biologists had not yet been able to reproduce with B-cells the factor-dependent growth of T cells that our colleagues T-cell biologists have been able to achieve.

FEEDER CELLS AND NEW MONOCLONAL ANTIBODIES YIELD MORE ROBUST B-CELL CULTURES

A possible explanation for our lack of success was the absence of feeder cells, which had become part of the T-cell culture system and proved necessary to allow for the expansion of human T-cell lines and clones. Meanwhile, Kevin Moore and his colleagues at DNAX, cloned a human cDNA coding for FcγRII/CD32 and found that FcγRII/CD32-transfected fibroblast cell lines could present monoclonal antibodies in a manner that allowed for cross-linking of the target molecule of the relevant cell (7, 8). More specifically, antibodies to the T-cell CD3 complex presented by these transfected cells together with IL-2 could induce prolonged T-cell proliferation (9). Thus, we wondered whether the presentation of monoclonal antibodies specifically directed at B-cell surface molecules in the presence of B-cell tropic cytokines would lead to the proliferation and expansion of B-cells.

By the end of the 80s, we, investigators from Schering–Plough/DNAX had cloned cDNAs encoding human GM-CSF (10), IL-4, IL-5 (5, 6), and FcgR/CD32 (8). We had also generated a number of monoclonal antibodies that would recognize B-cells including a CD40 antibody (11) and an anti-B7 antibody now known as CD86 (12). When Paolo de Paoli came to our lab to perfect his flow cytometry skills, he took a side project to refine methods for culturing sorted B-cells using both classical and new approaches, including the addition of a feeder-layer of CD32/FcγR-transfected cells as discussed above (9).

To this end, 96-well-plate microwells were first seeded with the irradiated fibroblast line. A few thousand B-cells were then added along with a few selected monoclonal antibodies with or without IL-2 or IL-4. Cultures were harvested 3–5 days later after a brief pulse with tritiated thymidine. It very quickly became apparent that the combination of the CD40 antibody Mab 89 (11) and IL-4 could induce unusually strong B-cell proliferation. The well-known CD40 antibody G28-5 made by Ed Clark and Jeff Ledbetter also proved highly effective in this system (13, 14). Curiously, IL-2 was unable to enhance CD40-induced B-cell proliferation, although it did enhance the proliferation of B-cells activated through their BCR. Furthermore, the fibroblast layer provided some feeder effect, as cross-linking the CD40 antibody on plastic was never as effective in inducing prolonged B-cell proliferation as presenting it with the CD32-transfected fibroblast.

NEW SYSTEM INCREASED B-CELL PROLIFERATION AND ENABLED LONG-TERM B-CELL CULTURE AND STUDIES OF B-CELL DIFFERENTIATION

The next critical experiment was to determine whether these culture conditions actually increased the output of B-cells. Indeed, it was very rewarding to find that the cultures made with CD40 antibody and IL-4 did generate more B-cells than were initially seeded. Subsequent experiments showed that with this new method we could establish proliferative B-cell cultures using relatively low numbers of B-cells (5,000 or less per well) compared to our previous purified B-cell cultures triggered with anti-BCR (20,000–50,000 per well). This

important finding, however, was not the end of the story, as we still had to show that this novel B-cell culture system would allow for the long-term growth (i.e., at least 3 weeks), of B-cells following splitting and feeding.

Some human B-cells harbor the Epstein–Barr virus (EBV), which, upon reactivation, can induce the generation of factor-independent lymphoblastoid B-cell lines. Thankfully, removal of the CD40 antibody and IL-4 quickly resulted in B-cell death. Furthermore, the factor-dependent B-cell lines failed to express EBNA-2 (Epstein–Barr Nuclear Antigen 2). These two findings led us to conclude that we were indeed generating factor-dependent human B-cell lines (15).

As is so often the case, novel methodologies enable us to address a whole new set of questions. We were thus wondering whether this new, feeder-layer/monoclonal antibody-based culture system would permit us to mimic many of the events happening in the germinal center where isotype switch, somatic mutations and differentiation into either memory B-cells or plasma cells are thought to occur (16). Indeed, Yong-Jun Liu and Ian McLennan showed that CD40-ligation prevents the spontaneous apoptosis of human centrocytes, which undergo antigen-driven selection within germinal centers (17). Our later studies and those from others demonstrated that CD40-activated B-cells could undergo isotype switch toward IgE when exposed to IL-4 or IL-13 (18, 19). Upon exposure to IL-10, CD40-activated B-cells switch toward IgG1 and IgG3 as well as IgA1 and IgA2 (20). The combination of IL-10 and TGFβ further enhances the IgA2 response. The critical studies led with the CD40 antibodies led to the cloning of it ligand (CD40-L), a molecule transiently expressed on T cells, by investigators at Immunex (21). The importance of CD40–CD40-L interactions in isotype switching in humans was further established when patients devoid of functional CD40-L were shown not to display switched isotypes (22–25). In further studies using CD40-L-transfected fibroblasts, rather than the combination of CD32/FcγR-transfected fibroblasts and CD40 antibody, we could show that CD40-ligation induces germinal center B-cells to differentiate into memory B-cells rather than plasma cells (26). Thus, this new culture method could robustly recapitulate key features of the germinal center and thus enable greater insight into the events leading to B-cell differentiation than previous approaches.

EXPLOITING THE NEW B-CULTURE SYSTEM FOR GENERATING HUMAN MONOCLONAL ANTIBODIES

A practical application of the CD40-system has been the efficient generation of human monoclonal antibodies. We eventually simplified the system to the point where peripheral blood cells (about 5,000 per well) from individuals displaying selected antibody specificities in their serum were simply cultured over CD40-L-expressing fibroblasts in the presence of exogenous EBV. Analysis of culture supernatants after 10 days eventually revealed the presence of antibodies of the desired specificity. This allowed us to generate large a number of monoclonal antibodies, such as those against the Bullous Pemphigoid Antigen1 (27), and against allergens, such as those from birch pollen (28). Nearly all these lines proved to be easy to generate; however, one set of autoantibodies gave us a hard time: autoantibodies against IL1α, which we previously found were expressed in about 10% of the healthy population. We generated dozens of B-cell lines producing anti-IL1α antibodies but had difficulty cloning them. We eventually isolated a single clone (29) that produced a high affinity ($Kd \sim 10^{-10}$) neutralizing monoclonal antibody. The reason why the cloning of the lines generating these monoclonal antibodies suggested to us that IL1α might have a critical role in B-cell expansion, an hypothesis we could not confirm. A strategic decision by the Schering–Plough leadership resulted in the discontinuation of our human monoclonal antibody program in 1994 around the time that the FDA rejected approval of the anti-sepsis monoclonal antibody, Nebacumab, also known as Centoxin® (30). Today, however, human monoclonal antibodies are a major success in the biotechnology and pharmaceutical world. Several methodologies to generate monoclonal antibodies are currently in use. These include: (1) engineering of mouse monoclonal antibodies, (2) generating monoclonal antibodies from animals whose Ig locus has been swapped with a human Ig locus, (3) the use of phage display libraries from pools of memory B-cells, (4) a method similar to ours where CD40-L cells were replaced by CpG (31), and (5) the isolation of single B-cells and the cloning of their heavy and light chains (32). Other have taken advantage of the power of the "CD40-system" to generate long-term B-cell lines that could be used as highly efficient antigen-presenting cells to generate autologous antigen-specific T cells for adoptive immunotherapy (33). The system can even permit the establishment of long-term porcine B-cell cultures (34). It is important to know that CD40 is expressed by a number of cell types other than B-cells. Most importantly dendritic cells express CD40 and are activated upon CD40-ligation (35). A few other cell types do as well [see Ref. (36)].

In conclusion, this study showed that it was possible to grow B-cells like T cells and opened up a path toward obtaining human monoclonal antibodies and understanding B-cell signaling. Before this work, many labs had tried to get human monoclonal antibodies from EBV transferred cells but the yields were small and most often low affinity IgM were generated. I am indebted in the numerous students and colleagues from Schering–Plough and DNAX who worked with me on these various projects. The support of Schering–Plough was essential for these studies.

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EPFL Innovation Park · Building I · 1015 Lausanne · Switzerland
T +41 21 510 17 00 · info@frontiersin.org · frontiersin.org